pH CONTROLLED DRUG RELEASE FOR DENTAL APPLICATIONS

By

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by

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To my parents Jeanne and Francis Wironen and to my uncle, the late Rev Aurelius Garnepy Better role-models could not exist

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pH CONTROLLED DRUG RELEASE FOR DENTAL APPLICATIONS

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A large proportion of the dental fillings replaced at present are revised because of the perceived presence of a recurrent caries under or adjacent to the restoration. Many of these perceived caries may not exist, while others may go undetected. This work describes the preparation of drug loaded polymer microspheres that sense the presence of the bacteria that cause caries by the associated presence of acid by products of digestion. These microspheres are designed to swell and release their antimicrobial drugs once the pH drops to a level that would normally cause caries.

The preparation of the microspheres as well as their loading with potassium fluoride, chlorhexidine digluconate, chlorhexidine dihydrochloride, chlorhexidine diacetate, and tetracycline hydrochloride are described. A detailed study of the controlled release behavior of fluoride as a function of polymer composition and pH is presented first. A study of the release kinetics of potassium fluoride, chlorhexidine digluconate, diacetate, dihydrochloride, and tetracycline hydrochloride as a function of pH in the same

polymer system is then presented. Additional studies of the swelling kinetics of chlorhexidine-loaded microspheres in various pH buffers are discussed with special reference to correlations with the controlled-release data. Finally, an experiment in which the microspheres are tested in an *in vitro* bacteria model that includes *Streptococcus mutans* is presented and discussed in detail.

CHAPTER 1
INTRODUCTION

Dental caries (cavities) remain a rampant problem.[1] In the first world, a constantly increasing sugar intake coupled with a diet of largely soft foods has led to a consistent problem with caries, despite wide public use of fluorinated water supplies and oral dentifrices.[1] A large portion of this problem stems not from primary caries, but from the recurrent variety below or adjacent to dental restorations. The perceived presence of recurrent caries adjacent to or under restorations is often the most important reason cited by dentists for the revision and replacement existing restorations.[2]

Among other bacteria, *Streptococcus mutans* has been identified as one causative agent in the caries etiology.[3] This bacterium, as well as the other primary bacteria involved in the formation of caries, is sacrolytic, and produces acid (lactic acid) as a waste product of digestion. This is the primary cause of dental caries, since these acid waste products lower the pH of the milieu around the enamel below the dissolution pH of the enamel (pH 5-5.5), causing it to dissolve.[3]

In the research presented here, one application of the materials developed is for the on demand release of bactericidal agents to kill the causative agents in the caries process. pH sensitive microspheres are described, that swell at or below pH 5.5 and release agents to kill the bacteria. In this application, microspheres could be placed into the cleaned and burred carious lesion before the application of a dental restorative material. If a caries does begin to form under the restoration, the microspheres will sense the presence of acid waste products from the microbes and release antibiotics. See figure 1.1.

Controlled drug release is a concept that has evolved over time from the growing concern of health practitioners over factors including patient compliance and the overdosing of patients to obtain a therapeutic threshold over a clinically useful period of time. The advantages of controlled drug delivery can be varied from on demand release to slow release over a long period of time. Figure 1.2 shows an example of the advantages of controlled-release over typical drug administrations. Depicted side by side are hypothetical, but realistic, serum levels of a drug administered orally or intravenously and by a controlled-release device. If this were a drug such as methotrexate, which is highly poisonous, exceeding the therapeutic by a small amount, as is routinely done with the intravenous or oral administration case, could cause drug levels to increase to toxic levels that could lead to death. Less than this dose, in many cases, would to lead to such rapid clearance of the drug that it would reach the therapeutic threshold for a clinically ineffective period of time [4].

The use of controlled-release technology can also allow for the local release of a drug well in excess of the therapeutic threshold, while keeping systemic levels very low. A final advantage to some systems is that they will release a drug only upon demand.

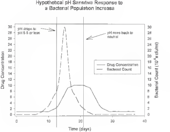


Figure 1.1. Hypothetical pH sensitive response to a bacterial population increase. The bacteria produce acid as a waste product of digestion, thereby lowering the pH. The drug is released at low pH, killing the bacteria and returning the environment to its normally neutral pH.

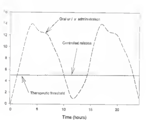


Figure 1.2 Hypothetical drug concentration with a controlled release device versus with an intra-venous (i v) injection or oral administration The controlled release device can be designed to maintain drug concentrations just above the minimum effective dose, avoiding overdosing the patient

The use of controlled release devices in dentistry to date has been limited. Caries preventative regimens including controlled release of fluoride, chlorhexidine, and tetracycline have been studied, but not widely employed [5-7].

In their 1994 publication "Broadening the Scope: Long Range Research Plan for the Nineties", the National Institute of Dental Research pointed out significant needs for preventative dentistry. They indicated the need for "... advanced microbial strategies against cariogenic bacteria that are long-lasting and do not increase the risk for opportunistic infections..." and "... remineralization strategies for controlling primary and recurrent caries in enamel and dentin." [8]

To address these needs, the study described within this dissertation attempted to apply pH controlled-release technology to the release of fluoride for remineralization therapy and chlorhexidine and tetracycline for antimicrobial therapy. The goal was to develop a technology that would release fluoride or an antimicrobial drug once the local environment became acidic, indicating the presence of cariogenic bacteria. The intended result of the work was to obtain a system that would retain the drug at a pH above 6.0 and release it at a pH of 5.5 or less to a sufficient amount to effectively kill any bacteria in the region and to remineralize tooth dentin. Figure 1.1 shows a graphic representation of the ideal release of fluoride or antimicrobial drugs in this application.

The more specific goals of this research were to:

(1) Develop methods of loading the mentioned drugs into lightly cross-linked copolymers (cross-linked with divinyl benzene (DVB)) of

diethylamino(ethyl methacrylate) (DEA) and methyl methacrylate (MMA) or ethyl methacrylate (EMA),

(2) Study the loading and controlled release as a function of polymer type and drug type,

(3) Test the biological efficacy of promising compositions in an *in vitro* bacteriology model with *S. mutans*

CHAPTER 2
BACKGROUND

Controlled-release technology is a broad field which covers a number of varied technologies. Controlled-release devices can range from simple blocks of polymer that contain dissolved drug to highly engineered osmotic pumps.[9] For descriptive purposes, controlled release devices can be categorized into a number of groups.

(1) Degradation or dissolution controlled devices include enteric coated aspirin, which dissolve at neutral pH after surviving the stomach, and devices based upon the degradation of biodegradable polymers, like poly(ortho-ester).[10]

(2) Monolithic systems are systems in which a drug is dissolved or dispersed within a nonbiodegradable polymer matrix. Drug delivery is accomplished through passive diffusion through the polymer to the surface.[11-13]

(3) Membrane-controlled systems employ a reservoir of liquid drug behind a semi-permeable membrane. Release of drug is accomplished through passive diffusion through the membrane.[14]

(4) Osmotic pumps and other mechanical devices usually combine a reservoir, a piston or valve, and a defined orifice to deliver drug at a controlled rate.[14, 15]

(5) Swelling controlled systems usually employ hydrogels that will absorb water under defined conditions causing them to swell. This swelling increases the size of the pores in the polymer matrix (mesh size) that in turn allows the loaded drug to escape.

Swelling controlled systems generally employ a glassy polymer that, when dry, has a T_g greater than the intended temperature of release (generally 37° C). During swelling, the solvent diffuses between the polymer chains. When a critical solvent concentration is reached, the polymer matrix is plasticized to the point where the T_g has reached the experimental temperature. At this point, the polymer undergoes a transition to a rubbery state.[16]

The kinetics of solvent penetration, and thus swelling, and release can frequently be described by a simple distillate of Fick's law of diffusion[17]

$$S=K_p t^n \tag{2.1}$$

where

S = the phenomenon of interest

K_p = a constant

t = time

n = an exponent

S in this equation, is a dummy variable which should be exchanged for **Q**, the fractional swelling, when swelling is of interest, or **M_t/M_∞**, the fractional release, when controlled-release is of interest. In the case of true Fickian swelling or release, n is equivalent to 0.5.[18-21]

Swelling penetrant diffusion and drug release from initially dry, glassy polymers is frequently more complicated than equation 2.1 implies. A significant deviation from the square-root of time dependance anticipated in Fickian diffusion is often observed.[16, 19-21] Glassy polymers undergo relaxation during solvent penetration. The characteristic time of relaxation may vary from very rapid to very slow relative to the characteristic diffusion constant for the solvent or drug. This leads to non-Fickian swelling and release behaviors.[22]

A classification scheme for diffusion and release in polymers has been proposed by Frish and Alfrey.[23-25]

Case I. This is the Fickian case. In this situation, the rate of polymer relaxation during swelling is much smaller than the rate of diffusion of solvent. In the case of release, the diffusion of the drug through the polymer is far slower than the rate of swelling. The value of n in equation 1.2 is very close to 0.5 for this case. $\mathbf{K}_g$ is proportional to the rate of polymer relaxation, in the case of swelling. In the case of drug release, $\mathbf{K}_g$ is proportional to the rate of drug diffusion in the polymer matrix.

Case II. This is a special non-Fickian case, commonly referred to as "zero-order" release, in controlled-release experiments. It describes a situation in which drug release is linear with time. The required swelling kinetics in this case are heavily affected by sample geometry. In this case, the rate of solvent diffusion into the matrix is much slower than the rate of polymer relaxation. A gel front progresses through the polymer as the solvent diffuses in. Just behind the front, the polymer is in a near-equilibrium state and just before the front, the glassy polymer contains virtually no solvent. In controlled-release cases, in addition to the above conditions, the rate of drug diffusion through the gel is also much more rapid than the rate of solvent diffusion into the polymer matrix, and thus more rapid than the rate of swelling. In this case, the value of **n** in formula 2.1 is 1.0. $\mathbf{K_s}$ is proportional to the rate of solvent diffusion, and thus to the velocity of the swelling front, in the case of swelling. In the case of release, $\mathbf{K_s}$ is also proportional to the rate of solvent diffusion and the velocity of the solvent front.

Anomalous diffusion. This final case describes the situation where the rates of solvent diffusion and polymer relaxation or drug diffusion are similar. Neither the swelling or the polymer relaxation or drug release is dominant, leading to compounded swelling and release behavior. Nothing can be said about the values of either **n** or $\mathbf{K_s}$ in equation 2.1 for this case.

Models of diffusion other than 2.1 have been developed [18-22, 26]. While all of them serve to further elucidate the phenomena occurring during various controlled release experiments, they frequently fall short of yielding fully accurate results. This is particularly a problem in the extremely complicated case of pH controlled-release studied here.

pH controlled-release can be considered a special case of swelling controlled-release. By creating a polymer that incorporates either mildly acidic or mildly basic monomers as part of its structure, the polymer can be made to be hydrophobic at a near neutral pH and then swell and become hydrophilic with a change in pH. Peppas and

Sassi have reported on the use of acrylic acid as part of a copolymer composition to lead to swelling at basic pH [17, 27] Batich, Siegel and Sassi, have reported on the use of 4-(or 2)vinyl pyridine or diethylamino(ethyl methacrylate) as part of a copolymer to lead to swelling at acidic pH [27-29] The polymers in all of these studies (with the exception those studied by Dr Sassi) incorporated the mentioned monomers into a hydrophobic copolymer with a lipophilic co-monomer like methyl methacrylate or styrene. In this way, the polymer remained hydrophobic and un-swollen until it reached its transition pH, at which it swelled rapidly and became hydrophilic.

In the case of drug loaded polymers of this type, the controlled release behavior becomes complicated It can be controlled by a number of factors These may include

(1) Choice of pH sensitive monomer and its pK_a

(2) Hydrophobicity of the polymer as a whole This is governed largely by the choice of the co-monomer and the ratio of hydrophobic co-monomer to pH sensitive monomer

(3) The cross-link density of the copolymer

(4) The buffering medium The factors governing the effects of the medium include the choice of buffering molecules and the ionic strength of the buffer

(5) Loading of the drug The presence of a drug in a polymer matrix can serve to accelerate or inhibit swelling, and so, as a result, release Additionally there is frequently an effect of loading rate on the release rate and swelling or release onset pH.

(6) Choice of drug. The hydrophobicity of the drug as well as its molecular weight, steric factors, and its charge all affect the rate of release.

Several authors have reported the effect of monomer choice on the transition behavior of pH sensitive hydrogels. Peppas and Brannon Peppas applied Florey-Rhener theory, rubber elasticity theory, and ionic interactions to the swelling of highly swellable, acidic, pH sensitive hydrogels. They found that the swelling in these theoretical hydrogels reached 50% of its maximum value at the pH value equivalent to the pK_a of the pH sensitive monomer.[30] Firestone and Seigel found that copolymers of methyl methacrylate (MMA) and diethylamino(methyl methacrylate) (DMA) which were loosely cross-linked, underwent a very sharp transition to a swollen state at just slightly below pH 6.6.[31] Batich reported that copolymers of styrene with 4-(or 2-)(vinyl pyridine) underwent a swelling transition at pH 5.0.[28]

Co-monomers and their ratios to the pH sensitive monomer were studied in detail by Seigel and Firestone. These authors found that in MMA/DMA copolymers, when the percentage of MMA was increased, the pH transition fell to a lower pH and the extent of swelling was reduced. They found that too much hydrophobic monomer (93%) eliminated pH-dependent swelling entirely. By exchanging the MMA for ethyl, butyl, propyl, or hexyl methacrylate, the equilibrium swelling decreased, but the transition pH changed very little. Copolymers including butyl, propyl, or hexyl methacrylate had such low glass transition temperatures that they were not glassy at 37° C, and so were not useful for pH controlled drug delivery. Seigel explained the decreased swelling and decrease in swelling transition pH. Simply stated, as the percentage of hydrophobic monomer increases, the amount of ionizable groups in the polymer decreases At the same time, the hydrophobic forces preventing swelling at high pH become stronger As the DMA groups ionize, the osmotic pressure inside increases Before a swelling transition can occur this osmotic pressure has to exceed the hydrophobic interaction forces that are preventing swelling. This means that a greater ionization is required, meaning a lower pH Additionally since the osmotic pressure cannot become as high, due to the decreased concentration of ionizable groups, the equilibrium swelling is decreased.[32] Batch found that this same phenomenon was observable in styrene/4-(and 2-)(vinyl pyridine) copolymers [28]

The effect of cross-linking density has been examined theoretically by Brannon Peppas and Peppas They found that an increase in cross-link density of acidic copolymers decreased the ultimate extent of swelling but did not change the transition pH [30] Batch found this same phenomenon to be true experimentally in styrene/4-(and 2-)(vinyl pyridine) copolymers.[28] Wei found that the same trend also held in MMA/DMA copolymers [33] Both Batch and Wei found that an increase in cross-linking density also resulted in a reduced loading of drug

The effect of buffers upon swelling has been examined theoretically by Brannon-Peppas and Peppas They found that the equilibrium swelling decreased with increasing ionic strength.[30, 34] By applying Donnan theory, Seigel and Firestone found theoretically that for MMA/DMA copolymers, there was a peak swelling at a fairly low ionic strength. The equilibrium swelling decreased when the ionic strength was brought above or below that peak. They confirmed the predicted trend for the increasing ionic strength experimentally.[35] These authors also found both theoretically and experimentally that the degree of swelling was different for citrate and phosphate buffers. The degree of swelling was decreased relative to that in phosphate buffer when in citrate solution.[5,38] Wei found similar phenomena experimentally, by examining the swelling of the same microspheres in buffers that contained acetic, butyric and phenyl acetic acid buffers. He found that both the degree of swelling and the transition pH were affected by the choice of buffering compound. With an increasing hydrophobicity in the buffering compound, both the transition pH and the maximum degree of swelling increased.[33]

In their studies of highly swellable copolymers of hydroxy(ethyl methacrylate) (HEMA) and acrylic acid loaded with theophyline, Brannon-Peppas and Peppas, found that there was little effect of percent drug loading on the release characteristics of the polymer drug system.[17] In the studies of Batich and Yan, however, the drug and the percent drug loading was found to strongly affect both swelling and release. In copolymers of styrene and diethylamino(ethyl methacrylate) (DEA) loaded with 9 amino acridine (AA) or sodium fluorescein, they found that AA loaded spheres swelled at a pH slightly above the swelling transition point of unloaded microspheres. These microspheres also released AA at this pH. This premature release was ascribed to AA increasing the osmotic drive for solvent to enter the microspheres. Additionally, the loaded microspheres were found to swell at a slower rate than unloaded ones at pH's below the swelling transition. The reduced rate of swelling was ascribed to the fact that AA is a cationic drug that would compete with the polymer for hydronium ion during swelling, thereby reducing the net concentration of hydronium available to induce swelling. A decrease in swelling half-life was correlated with an increase in percent loading for these microspheres. For sodium fluorescein (a highly charged dye) loaded spheres, the behavior was much more complex, and more difficult to explain. At pH 7 un swelled microspheres released fluorescein, while at pH's below the swelling transition, the microspheres did not release. While AA is a cationic drug, fluorescein is an anionic drug. The observed phenomena are likely a combination of improved solubility of the drug at pH 7 over lower pH, due to increased ionization, and binding of the anionic drug to the cationically charged polymer matrix.[37]

Yan loaded the same microspheres with AA and fluorescein in the study mentioned above. He observed different release behaviors for the two cases.[37] Little other work has been done loading different drugs into the same polymer matrix. Most other researchers concentrate on the study of one drug, or on one family of very similar drugs.[17, 29, 32, 35, 38] It remains a challenge to conduct a study comparing the release and swelling behaviors of the same polymer loaded with different types of drugs.

Three different basic drug types were studied in this work, namely, three chlorhexidine derivatives, tetracycline, and fluoride. The beneficial effects of fluoride in dentistry have been widely discussed.[39-43] They include bacteriostatic effects and the formation of acid resistant, and hard fluoro apatite on the enamel surfaces.[5, 36] The remaining two drug types mentioned here may not be as familiar, but are also widely used in dentistry. Tetracycline is routinely used, and has been shown to be effective as part of therapy for periodontal disease.[7] Chlorhexidine, in its various forms is used in the form of mouth washes and varnishes, and is being explored for use in controlled release devices both for caries prevention, and for vigorous treatment of caries and periodontal disease.[44, 45]

Figure 2.1 shows the chemical structures of chlorhexidine digluconate, chlorhexidine diacetate and chlorhexidine dihydrochloride. The molecular weight of chlorhexidine is 504 Daltons, not including the attached counter-ions. It has been shown to form nearly insoluble salts with, among other things, phosphate, sulfate, and chloride ions.[46] Its minimum bactericidal concentration for established *S. mutans* plaques, was measured by Slee to be approximately 3.2 mM (1600 ppm).[44] The minimum inhibitory concentration for *S. mutans* in broth culture was measured by Drake to be 0.74 μM (0.37 ppm).[47]

Figure 2.2 shows the chemical structure of tetracycline HCl. Its molecular weight is 443 Daltons. As can be seen from the figure, the basic chemical structure, regarding approximate size and charge is very similar to that of chlorhexidine. The minimum inhibitory and bactericidal concentrations for *S. mutans* with tetracycline are not known to the author.

No figure of fluoride is shown for obvious reasons. This is a highly electronegative ion with a negative charge. The atomic weight of fluoride is 18 Daltons

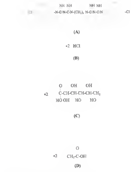


Figure 2.1 The structure of chlorhexidine (A) and its ligands, hydrochloride (B), gluconate (C) and acetate (D)

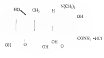


Figure 2.2 The structure of tetracycline hydrochloride

Fluoride has been inserted into various controlled-release devices through the years. Primary among these are the glass-ionomer cements [48-59] These cements generally employ an dental ionomer such as copolymers containing acrylic acid with a glass that contains large amounts of fluoride, sodium, and a multivalent ion for cross-linking like aluminum [48, 49, 60] The ionomer is slowly cross-linked by the ions on the surface of the glass particles, and the fluoride is free to elute from the cement by passive diffusion. These cements have had considerable success in applications where long-term stability is not a concern.[48, 49, 51, 53] Another area where fluoride controlled-release has been employed is in an orthodontic cement developed by Rawls [41]. These cements employ an aminated resin to which fluoride ion has been attached. Release is by ion exchange with the salivary fluid. The commercial and clinical success of these products has yet to be proven.[39, 56, 61-63] Each of these fluoride controlled-release systems relies on simple ion exchange and provides a continuous release of fluoride.

Controlled release of antibiotic for dentistry has been studied by a number of authors. Shen *et al.* studied the *in vitro* efficacy of the pH controlled release from MMA/DEA microspheres of nystatin and clotrimazole on *Candida albicans*. They found that the microsphere preparations effectively limited the growth of the studied organisms [64] Kozlovsky, *et al.* studied films composed of methacrylic acid and chlorhexidine digluconate in beagle dogs. This system proved to be biodegradable, easy to apply to the teeth, and controlled supra gingival plaque levels well [65] Mirth *et al.* studied chlorhexidine release from a monolithic device made from HEMA/MMA mixed with chlorhexidine diacetate powder, and coated with a membrane of HEMA/MMA These authors observed near perfect 0-order release *in vivo* [6]

Tetracycline controlled release devices have been used in the form of fibers. Goodson, *et al.* found that tetracycline loaded biodegradable fibers reduced the levels of micro flora in some periodontal pockets to near zero after a month in place [66] Olanoff Koinis, and Anderson developed a membrane-controlled tetracycline delivery device based on HEMA and MMA based copolymers They were able to show 0-order release kinetics for over 4 months *in vitro* [7]

CHAPTER 3
MATERIALS AND METHODS

Synthesis of Microspheres

The microspheres used in these studies were pH sensitive varieties composed of random copolymers of methyl methacrylate (MMA) or ethyl methacrylate (EMA)and diethylamino(ethyl methacrylate) (DEA) Styrene and 4 vinyl pyridine copolymer microspheres were used in the studies of swelling measurement techniques, which will be described later in this text

The mechanical stirring, heat treatment, and apparatus used for each of these varieties of microspheres was the same The aqueous phases did differ, however

DEA/MMA or DEA/EMA Microspheres

These microspheres, except where noted in the text, were kindly supplied by Dr William Toreki The method of synthesis used follows.

The aqueous phase. The aqueous phase in the suspension polymerization was made by adding 0 590 g. bentonite (Fisher, lot 914323A), 0 16 g. hydroxyethyl cellulose (HEC) (Aldrich, lot 16111CZ) and 25 3 g. NaCl (Fisher, lot 861673) to 120 ml distilled water (DH20) and stirred rapidly (about 300 rpm) for at least 10 minutes with the agitator shown in figure 3 1

Monomer and initiator preparation Impurities and inhibitors were removed from stock monomers by either distillation or washing with base.

Divinyl benzene (Polymer Laboratories, Windham NH, lot 6-46-1) was mixed 50 50 (v/v) with 1 M NaOH (Fisher, lot 7129926) in a separatory funnel The funnel was shaken and burped several times and then the materials were allowed to separate. The aqueous phase was drawn off and the oil phase was washed a second time in a similar manner Clean monomer was stored at 2-4°C

Methyl methacrylate (MMA) monomer (Aldrich lot 05018TZ) was vacuum distilled using the apparatus displayed in figure 3 2 Vacuum was maintained at 30" Hg while a very light stream of argon or nitrogen was allowed to bubble through the monomer The temperature at which the monomer boiled varied from 38-45°C

Ethyl methacrylate (EMA) (Aldrich lot 04025LN) and 2-diethylamino(ethyl methacrylate) (DEA) (Aldrich, lot 13126DG) were distilled in exactly the same manner as methyl methacrylate The boiling points of these monomers were 45°C, and ~80°C respectively 2-2'-Azo-bis-isobutyronitrile (AIBN)(Aldrich) was recrystallized from methanol

Monomer mixture preparation Monomers were weighed out into vials on an analytical balance. Monomer molar ratios will be indicated for each separate batch Monomers were mixed thoroughly in an Erlenmeyer flask and AIBN was added in a finely crushed form in approximately a 1 300 w/w ratio (initiator/monomer) The

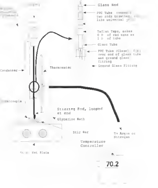

Glass Rod
PVC Tube connec two rods together like universal jo
Teflon Tape, makes O.D. of rod same as I.D. of tube
Glass Tube
PVC Tube (Clear) fi over end of glass tube and ground glass fitting
Ground Glass Fitting
Condenser
Thermometer
Thermocouple
Stirring Rod, looped at end
Glycerine Bath
Stir Bar
To Argon or Nitrogen
Temperature Controller
Stir Hot Plate
70.2

Figure 3.1: Polymerization apparatus

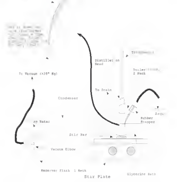


Figure 3.2: Monomer distillation apparatus. The argon flowing through the modified pipette carries the vapor and agitates the monomer but does not greatly affect the degree of vacuum attainable.

initiator was allowed to dissolve thoroughly (by swirling) in the monomer mixture before addition to the aqueous phase.

Polymerization. The aqueous phase was allowed to heat up to 70°C while inert gas (nitrogen or argon) was pumped through the chamber Temperatures were maintained to within ±2°C by using a digital temperature controller (Honeywell, DC2003, Clearwater, FL) , as noted in figure 3 1 When the proper temperature was reached, the monomers were added to the reaction chamber in one fell swoop, while the agitator was stirring at 250-350 rpm The reaction was allowed to continue at 70°C for 5 6 hours and then the temperature was increased to 80°C for 1-2 hours to complete consumption of residual monomer

Microspheres were isolated from the aqueous phase and dross by allowing them to settle in a tall (2 L) graduated cylinder The dross which generally had small air bubbles in it would rise to the top where it could be poured off while the microspheres would settle After settling, the liquid was decanted off, and the microspheres were rinsed in several changes of tap water Finally, spheres of certain size ranges were isolated by pouring an aqueous suspension of them through a series of sieves Microspheres were dried in a 60°C vacuum oven at 30" Hg for 24-48 hours

Microspheres with Intrinsically Loaded Fluoride

Intrinsically loaded microspheres were produced in a manner exactly like those that were not loaded with fluoride salt, except that mixing and pre-polymerization steps were added, and that the aqueous phase was saturated with the fluoride salt in order to prevent solvent separation of the fluoride All of these intrinsically loaded microspheres were produced by the author

Potassium fluoride (Acros lot B0029462) or sodium fluoride (Fisher, ACS, lot 732824) was added to the bentonite/HEC aqueous phase (described previously) to amount to at least 8% by weight The undissolved fluoride particles were obvious at the bottom of the reaction chamber if stirring was stopped indicating a saturated solution

Potassium fluoride (Acros, lot B0029462) or sodium fluoride (Fisher, ACS, lot 732824) powder was forced through a 38 μm sieve This powder was then added to a monomer mixture composed of 20% DEA, 3% DVB 77% MMA (mole %) and 1 300 w/w initiator monomer AIBN in an Erlenmeyer flask to total 10-15% w/w fluoride salt The monomer/drug mixture was purged with argon, a stir-bar was added and then capped with a cork stopper A 1 ml sample of this mixture was added to a 20 ml serological tube purged with argon and capped then placed into a 70°C glycerine bath, unstirred. Exactly 10 minutes later, the remaining monomer/drug mixture was suspended by means of a clamp above the center of the glycerine bath and stirred rapidly with the magnetic stir plate under the bath (see figure 3 1) The 1 ml monomer mixture was monitored closely, by manual shaking to determine when it had reached the gel point Once this monomer had gelled, the remaining monomer was added to the aqueous phase in the reaction vessel and allowed to react as a normal batch of microspheres as described previously All subsequent processing and separation was exactly as previously described for standard microspheres

Synthesis of Styrene 4-Vinyl Pyridine Microspheres

Except where noted, these microspheres were kindly synthesized and provided by Dr Jun Yan. They were made with very similar methods to those outlined above, with the exception of the aqueous phase

The aqueous phase. The aqueous phase used in these preparations was made by adding 24 ml of a 5% aqueous solution of poly(vinyl alcohol) (M_s=15-27 KD) (PVA) (Airvol 205, Air Products, USA, lot 02090839) to 96 mL of distilled water The aqueous phase was allowed to heat to 70°C while stirring in the polymerization apparatus before adding the monomer mixture

Monomer preparation. Monomers and the initiator were prepared in a manner similar to that described above

Styrene monomer (Aldrich lot 05031SN) was distilled at 40 °C using the standard distillation apparatus pictured in figure 3 2. The 2-vinyl pyridine monomer (Aldrich lot 03926LY) was also vacuum distilled at 58-59°C All other source materials were treated as described in previous sections

Polymerization Monomers were mixed as described in previous sections and added to the reaction chamber once the aqueous phase had reached 70°C and had been purged with nitrogen or argon

The polymerization was allowed to continue at 70 ± 2°C for 5-6 hours while stirring at 250-350 rpm. The temperature was raised to 80°C and the reaction was allowed to continue for another 2 hours while still stirring Microspheres were isolated by allowing them to settle from the aqueous phase in a tall cylinder as described in previous sections. They were sieved and dried as described for other micro sphere types.

Synthesis of Fluoride Loaded Microparticles

Fluoride loaded microparticles were produced by bulk polymerization of DEA MMA, and DVB under rapid agitation with particulate KF 2.38 g DEA(Aldrich, lot 13126DG) 0.20 g DVB (Polymer laboratories, Windham, NH, lot 6-46-1), and 5.07 g MMA (Aldrich, lot 05018TZ) were combined with 0.024 g AIBN (recrystallized by C Sakezles) and purged of oxygen by rapidly bubbling argon through the mixture for 5 minutes.

KF (Acros lot B0029467) powder was forced through a 38 µm mesh with a palate knife and 1.04 g of the resulting powder was added to the monomer mixture. This monomer/drug mixture was purged a further 5 minutes with argon and then added to the reaction chamber depicted in figure 3.3. At the time of the addition of the monomer to the reaction chamber the outside bath temperature was 70.0 °C.

The polymerization was allowed to proceed at 70 °C under agitation at 1400 rpm until the gel point was reached and the polymer had become relatively hard (1.5 hours). After this point, the polymerization was allowed to continue without stirring at 70 °C for a further 7.5 hours. The temperature was increased to 80 °C and the reaction was allowed to proceed for a further 3.5 hours.

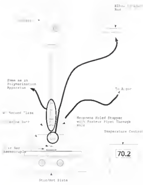


Figure 3.3 Bulk polymerization apparatus for producing KF loaded microparticles.

To facilitate removal of the polymer from the reaction flask, xylene was added to the reaction chamber to fill it completely (about 20 ml.). After 10.5 hours, the swollen polymer was removed from the reaction chamber and transferred to a 50 ml centrifuge tube. More xylene (30 ml.) was added and the polymer was set to spin on a Labquake® (Barnstead Thermolyne, Dubuque, IO) sample rotator (30 rpm) to tumble for 24 hours.

Excess xylene was removed from the swollen polymer by centrifuging it in a Dynac II centrifuge at maximum power (about 3,000 rpm.) for 5 minutes and then decanting. The swollen polymer was reduced to microparticles by mashing it with mortar and pestle. Approximately 20 ml more xylene was added to the powder and the mixture was set to tumble for 2 more hours. After this last wash, the sample was centrifuged and decanted again.

Microparticles were dried in a hooded oven (Blue M Fractionaire® Blue Island, IL) at 60 °C for 4 hours and then further dried in a vacuum oven at 60 °C and >30" Hg for 2.5 hours. They were then and allowed to tumble in 40 ml distilled water for 1 hour and then dried again in the vacuum oven at room temperature (>30" Hg). A second aqueous wash and dry was performed.

Micro Particle and Microsphere Size Determination

Microparticles and microspheres were attached to a piece of Scotch® tape (3M Co. Minneapolis MN) and placed on aluminum SEM stud. The samples were coated with carbon in a sputter coater, and scanning electron microscopy (JOEL) was conducted to yield low magnification images of fixed microspheres or microparticles (100x at 2kV)

Individual microspheres were measured with a ruler, and marked off on the SEM print so that they were not counted twice. The scale bar on the print was also measured so that sizes in millimeters could be converted to microns. Microparticles were sieved to eliminate particles over 150 µm and measured in a similar manner except that the measurement recorded was that of the longest linear measurement that could be obtained (e.g. corner to corner diagonally on square particles). The average and standard deviation of the sizes were reported

Loading of Microspheres with Antibiotics

The same basic technique, with few notable exceptions, for loading microspheres with drug was used throughout these studies. Namely, the drug was loaded into the spheres by first dissolving it into a good solvent for both the drug and the polymer, and then soaking the polymer in this solution. Diffusion, in theory would drive the low molecular weight drug into the polymer

The difficulty with using these extrinsic loading techniques is finding a solvent/polymer/drug system that is compatible. This was the thrust of a large amount of the work described below

Drug and drug solution preparation. Chlorhexidine digluconate is normally supplied as a 20% aqueous solution by the distributor (Sigma Chemical Company). Since methanol solutions were required for this work, it was necessary to first dry this solution out and then re-dissolve it in methanol. The drug was kept chilled at 4°C both before and after drying. Drying was accomplished by placing it into a 72 mm Pyrex® crystallizing dish, covered with gauze attached via a rubber band hooped around the edge of the dish. This dish was then placed in a vacuum oven (Napco Model 5831 Tualtin, OR) at room temp. 30" Hg for 72 hours. Final drying was achieved by raising the temperature to 60 °C for 4 hours. The gauze top served to prevent the foam-like final material from boiling over the top. The final material was crushed with a spatula and poured into dark-glass vials for storage at 4°C. Solutions of this drug were made by mixing appropriate amounts of methanol and drug (by weight) and placing them into a 25 ml round bottomed flask (Pyrex® brand) with a closed column of Drierite® (W.A. Hammond, Drierite, Xenia, OH) attached to the ground glass opening and stirring with a stir bar and magnetic stir plate for 2-4 hours. It was noted that if the Drierite® column was not used, a much lower solubility was achieved, and the solutions were frequently cloudy.

Drug solubility. Polymer/solvent compatibility experiments had been done by previous researchers[28, 29, 37], and determined that all polymers studied here were highly swollen in methanol, ethanol, tetrahydrofuran/water mixes, xylene, toluene, and similar solvents. With these solvents in mind, the solubility of un-derivatized chlorhexidine, and its diacetate digluconate, and dihydrochloride salts was determined by standard percent salts analysis of clear liquors lying over excess solute. All samples were prepared with a large excess of solute and agitated for more than 12 hours before analysis Liquors were filtered through a 0.2 µm syringe filter (Corning Glass, Corp., Corning, NY)

Un-derivatized chlorhexidine was virtually insoluble in all solvents except acetic acid, due to the conversion to chlorhexidine diacetate and in some THF/water solutions It was not more than 8% soluble in any solvent tested Derivatized chlorhexidine, however was found to be highly soluble in methanol and some THF/water solutions

Effect of Drug Solution Concentration on Loading. Experiments that were conducted using nearly saturated solutions of chlorhexidine digluconate in methanol yielded spheres with a lower than expected drug loading percentage. This lower than expected loading could be an effect of osmotic pressures or other potential concentration-dependant phenomena As a consequence, microspheres were loaded using various concentrations of drug in solution in order to determine the dependance of percent loading on drug concentration.

A stock concentrated solution of chlorhexidine digluconate (Sigma lot 963403 dried as previously described) in methanol (Fisher Optima grade lot 963403) was prepared by dissolving 4 0 g. chlorhexidine digluconate in 6 7 g methanol using methods described above (end concentration 39 2 % w/w) Portions of this stock solution were diluted down with methanol to yield solutions containing 29 4, 23 1, 14 8 and 8 0 % w/w chlorhexidine Approximately 0 06 g of microspheres were added to 1 ml of each of these five solutions After 48 hours, the microspheres were filtered out of the solution using a 3 ml 0 2 µm centrifuge filter tube (MSI, Fisher Scientific, Atlanta GA) for 10 minutes at max speed in a Dynac® centrifuge (Clay Adams Inc. USA) The microspheres were rinsed with tap water and centrifuged again for 10 minutes. Drying was accomplished in a vacuum oven (Napco, model 5831, Tualatin, OR) at 30" Hg and room temperature for 48 hours After drying the microspheres were rinsed twice by washing in tap water and then centrifuging. Microspheres were again dried under vacuum at room temp for 48 hours

Dry microspheres were observed under a stereo microscope (Olympus, Tokyo Japan) to determine if any drug crystals remained on the surface Upon confirmation that no drug crystals remained on the surfaces, microspheres (approximately 01 g. each type) were placed into polypropylene vials and methanol was added (approximately 5 ml) For each of the 5 batches of microspheres 3 samples were produced in this way After 7 days, the methanol solutions above the microspheres were analyzed for chlorhexidine digluconate content by UV/Vis spectroscopy (Shimadzu, Tokyo Japan) at 259 nm A blank solution of methanol was used, and methanol based standards were employed

Loading of Microspheres with Chlorhexidine for Experimental Use

The microspheres used in the controlled release and bacteriology experiments were loaded using the best methods determined by the previous analysis to maximize the percent loading of drug

Loading of microspheres with chlorhexidine digluconate. Three compositions of microspheres were loaded each in a solution of dry methanol containing 24 ± 0 0 % chlorhexidine.

Drug solution was prepared as described above by mixing 13 7 g chlorhexidine digluconate (dry) (Sigma lot 65H0427) and 46 39 g methanol (Fisher Optima lot963403) for 48 hours at room temperature The final concentration was determined (to account for evaporation) to be 24 ± 0 0 % by standard E P A percent salts analysis Approximately 1 to 1 4 g of microspheres were added to about 15 ml of drug solution Batches T4 T40, and T51 were loaded in this manner (see table 4 1 for batch composition).

Microspheres were rotated at approximately 30 rpm. on a Labquake® sample rotator (Barnstead Thermolyne, Dubuque, IO) for 3 days Microspheres were poured through hand-made micro-sieves (see figure 3 5 a) and rinsed with distilled water Drying was accomplished in a vacuum oven at 30" Hg, room temperature for 24 hours Microspheres were rinsed again, 5 times, using 5 ml of distilled water for each wash. They were allowed to drain in the micro-sieve between each wash. A second drying cycle in the vacuum oven at 30" Hg and at room temperature was conducted for 48 hours At this point the microspheres were examined for the presence of surface crystals under reflected light microscopy (Olympus Tokyo Japan) A third rinsing and drying step done as previously described was used to remove these residual drug crystals Light microscopy was conducted again to confirm that no drug crystals were present outside of the microspheres

Table 3.1: Polymer Compositions (Compositions are stated in molar monomer feed percentages.)				
Batch #	**%MMA**	**%EMA**	**%DEA**	**%DVB**
T4	0	91.6	6.6	1.8
T40	77.0	0	19.9	3.1
T41	77.0	0	19.9	3.1
T51	0	79.1	18.3	2.6
Microparticles	77.0	0	20.0	3.0

Loading of microspheres with chlorhexidine diacetate. Microspheres were loaded with chlorhexidine diacetate in a manner similar to that used to load them with chlorhexidine digluconate.

A 23% solution of chlorhexidine diacetate (Sigma lot 85H0151) was made in methanol (Fisher Optima, lot 76H0853) and shaken briefly to complete dissolution. Into a 15 cc centrifuge tube, 1.5 ml of drug solution and 30 g of T40 microspheres were added. Samples were rotated cleaned and dried exactly as described above.

Loading of microspheres with chlorhexidine dihydrochloride. Again, the method of loading was similar to that previously described. The concentration of drug in solution was lower, however, due to the lower solubility of this salt in methanol.

A saturated solution of chlorhexidine dihydrochloride (Sigma lot 76H0853) was made by adding 6 g of the drug to 1.8 g of methanol (Fisher Optima, lot 76H0853). Mixing was done on a Labquake® (Barnstead Thermolyne Dubuque IO) rotator inside of a 4° C refrigerator (chlorhexidine dihydrochloride is unstable in solution at room temperature) for 24 hours. The solution was filtered through a 0.1 µm syringe filter

(Corning Glass Corp Corning NY) and added to 25 g of T40 microspheres in a 15 cc centrifuge tube Loading, washing, and drying were accomplished exactly as previously described

Tetracycline HCl Loaded Macrospheres

Microspheres were also loaded with tetracycline HCl in order to allow comparison of the release of two similar antibiotics The methods used for this loading were exactly comparable with those described for loading various chlorhexidine salts.

A solution of 0 8 g tetracycline HCl (Fisher lot 931936) mixed with 7.2 g methanol (Fisher Optima lot 963403) was placed into a 15 cc centrifuge tube and shaken until dissolved 0 25 g T41 microspheres were added to the solution and the mixture was placed on a Labquake® sample rotator (Barnstead Thermolyne, Dubuque IO) inside of a 4° C refrigerator for 7 days Rinsing and drying were accomplished as previously described

Percent Loading Analysis of Antibiotic Loaded Microspheres.

Microspheres were analyzed for percent loading by simply reversing the process of loading Extraction of the drugs in an excellent solvent for both the micro sphere and the drug leads to a simple analysis of the solutions

Between 10 and 20 mg of each type of micro sphere namely chlorhexidine digluconate diacetate dihydrochloride and tetracycline hydrochloride loaded microspheres was placed into a 15 cc centrifuge tube Approximately 3 ml of 0 05 M aqueous citric acid was added to each tube. Tubes were placed on a sample rotator for 7 days in the dark.

The microspheres were allowed to settle out of solution and the concentration of drug in solution was determined by conducting UV spectrophotometry (Spectronics Genesys®, Spectronics, Rochester, NY). Water based blanks and standards of each drug were used. Analysis for chlorhexidine was done at 256 nm. Analysis for tetracycline HCl was done at 358 nm.

Fractional loading was found by computing the following

$$L = \frac{C * A}{1000 * W} \tag{3.1}$$

where

L = Fractional loading (L*100=%loading)

C = Concentration of drug in solution in ppm

A = Mass of acid added at the start of extraction in g

W = Mass of microspheres extracted

Loading of Microspheres with Fluoride and its Salts

Fluoride was loaded into microspheres, after the appropriate choice of salt, by methods as previously described. The challenges were finding the proper fluoride salt to load, determining the proper concentration of salt in solution and using the proper drying technique to obtain maximum loading.

Fluoride Salt Solubility

Sodium fluoride and potassium fluoride were studied as possible drugs for loading. The published solubility of sodium fluoride according to the Merck Index is 20% w/v [67]. The solubilities in both water and methanol were measured by standard E.P.A., gravimetric % salts analysis. From these data, it was determined that there was an error in the published values for sodium fluoride. Potassium fluoride showed excellent solubility in both methanol and water (see table 3.2).

Potassium fluoride was chosen as the drug of choice in these loading experiments because it showed much higher solubility in methanol while also showing lower solubility in water than sodium fluoride. This will potentially allow for both greater loading and slower release into aqueous media.

Table 3.2: Solubility of fluoride salts in selected solvents (% w/w)

Salt	Water	Methanol	90% Methanol	Ethanol
NaF	3.8±.01	2.1±0.0	1.0±0.0	0.1±0.0
KF	2.4±0.4	13.0±2.1	12.8±5.4	3.1±0.2

Swelling of Microspheres in KF/Methanol Solutions

The swelling of microspheres in KF/methanol solutions was measured to determine the optimum solution concentration for loading of drug into microspheres

A modified version of the digital light microscopy technique (described later in this text) was used to make these measurements. One each of lot 0121A microspheres (70 MMA, 29% DMA, 1% DVB) were placed at the bottom of a well in a 96 well polystyrene plate (model 25850, Corning Glass, Corning NY) by using fine forceps and a stereo-microscope (Olympus, Tokyo, Japan). Rows of 5 wells with one micro sphere in each well were used for each solvent/drug mixture. A stock solvent drug mixture was made by adding 1.25 g. KF (Acros lot B0029467) to 9.18 g. methanol (Fisher, Optima grade lot 963403) in a 50 ml polypropylene centrifuge tube and stirring mildly for 2 hours with a stir bar, upside down on a stir plate (end concentration 12.00 % KF w/w). The stock solution was then diluted with methanol to make solutions containing 9.8, 6.8, 4.6, and 2.2 % w/w KF. 200 µl of the appropriate solution was added to each of 5 wells. A control of 100% methanol was run along side of these solutions. The cells were covered with a layer of Parafilm® (American National Can, Neenah, WI) and then the cover of the well plate was forced on top of this and warmed with a hair drier to effect an air-tight seal.

The microspheres were photographed immediately on an inverted microscope (Baush and Lomb, Photo Zoom®) at 80x using Kodak Gold 100 film. After 4 days, the microspheres were photographed again. Photography was used instead of digital microscopy only because the digital camera was not available.

Photographs were all printed with a fixed magnification. The frame edges were printed in the end photograph to ensure confirmation of the fixed magnification. Individual photographs were analyzed side by side with the digital camera equipped with a light stand and a zoom lens (Nikon, Tokyo, Japan) Images were acquired of the un-swelled and swelled spheres side by side and their sizes in pixels compared

A theoretical percent loading curve was made by multiplying the drug concentration by the percent swelling. This was assumed to be the maximum possible loading at that drug concentration. All loading experiments from this point on were conducted using a drug concentration in solution near the maximum of this theoretical loading curve.

Drying of Microspheres After Loading

It was noted in a series of experiments, that the amount of fluoride loaded into microspheres was well below the amount predicted by the swelling studies One possible explanation was that the drug was being carried out of the microspheres during room temperature evaporation of solvent and being washed away To investigate this possibility, two alternative techniques to remove the solvent from the microspheres were tested

A stock solution of 27 g methanol (Fisher Optima grade, lot 963403) and 3.3 g KF (Acros lot B0029467)(end concentration 10 9% w/w) was made Approximately 0 5 g of T41 microspheres were added to this stock solution in a 50 cc polypropylene centrifuge tube The vial was rotated on a Labquake® sample rotator (at approximately 30 rpm) (Barnstead Thermolyne Dubuque IO) for 5 days at room temperature Excess solution was drained from the vial and the micro sphere batch was split roughly in two

Freeze Drying One half of the spheres were frozen in liquid nitrogen and freeze dried at less than 30 µm mercury vacuum in a Vitris freeze drier (Vitris, Gardiner NJ item no. 255265) for 24 hours After removal from the freeze drier, the microspheres were placed into a vacuum oven at 63 °C, 30" Hg., for 24 hours (to anneal the polymer matrix forcing collapse of freeze-dried structure) Once spheres were removed from the oven, they were rinsed with distilled water (about 15 ml) and place into the vacuum oven to dry 24 hours at room temperature, 30" Hg

Solvent/Non-solvent extraction Approximately 10 ml of Xylenes (Fisher ACS grade lot 943012) was added to the second half of the microspheres The spheres were agitated by placing the vial on a Labquake® rotator for 1 hour and then were decanted and dried in the vacuum oven at room temperature, 30" Hg 24 hours Xylene extracted microspheres were rinsed and dried again under the same conditions.

Percent Loading Analysis

Each batch of microspheres was analyzed by placing between 10 and 20 mg of microspheres into a 15 cc polypropylene centrifuge tube and adding 1-3 ml of methanol (Fisher Optima, lot 963403). The weight of methanol and microspheres was carefully recorded These samples were rotated for 7 days on a Labquake® sample rotator at approximately 30 rpm

After 7 days the spheres were allowed to settle out of solution, and the supernatant was drawn off and weighed into a second polypropylene tube. The supernatants were dried in an 80°C oven for 3 days. The residue was reconstituted with 2 ml of TISAB

(Fisher Scientific, Atlanta, GA) and measured for fluoride content with an ion specific probe as previously described.

Fractional loading was computed from the following formula

$$L = \frac{C * S}{500 * T * W} \tag{3.2}$$

where

L = Fractional loading of microspheres

C = Concentration of drug in ppm

S = Mass of methanol used for extraction in g

T = Mass of supernatant that was evaporated in g

W = Mass of extracted microspheres in mg

Percent loading is simply computed by multiplying fractional loading by 100.

Loading of Microspheres With KF for Experimental Use

Due to the high swelling of microspheres in 10-12% KF/methanol solutions, microspheres for experimental use were loaded in this concentration of solution. Due to the superior performance of freeze-drying and annealing treatments, these were employed in the loading as well.

In a polypropylene jar with a stir bar, 7.2 g. of KF (Acros lot B0029467) and 60.4 g. methanol (Fisher Optima grade, lot 963403) (end concentration 10.7 % w/w) were mixed with rapid stirring. About 2 g. of microspheres were added to 22-25 ml. KF solution in a 50 ml. polypropylene centrifuge tube. These tubes were agitated on a

Labquake® sample rotator for 6 days at room temperature. Excess solution was removed by decanting the solution through a 70 µm mesh (Nitex®, Tetco, Inc, Briarcliff Manor, NY) The microspheres were immediately frozen in liquid nitrogen and then lyophilized for 24 hours in a home-made freeze-drier comprised of a Labconco® freeze-flask and cap connected directly to a 200 l/min vacuum pump (Fisher Maxima, Fisher Scientific) Microspheres were annealed in a vacuum oven at 65°C, 30 ' Hg for 24 hours Upon removal from the vacuum oven, the samples were rinsed with more than 50 ml water and then dried at room temperature, 30 ' Hg, for 24 hours. The lack of crystals on the surfaces of the microspheres was confirmed under a dissecting microscope as previously described. Percent loading was determined as previously described.

Measurement of the Swelling of Microspheres

The microspheres used in these experiments exhibit very slight swelling Standard techniques like that described by Pepper[68] proved too insensitive and imprecise for these measurements As a result a high precision high sensitivity method for measuring polymer swelling was developed and compared with Pepper's technique

High Precision Measurement of the Swelling of Microspheres[69]

Sample cell preparation Poly(styrene-*co*-[2-vinyl pyridine]-*co*-divinyl benzene) microspheres were used in these experiments The molar ratio of the feedstock for these microspheres was 9/90/1 (styrene, 2-vinyl pyridine, and divinyl benzene, respectively) The size ranged from 100 to 400 µm

Cel-Tek Grid Slides™ were coated with a thin coating of household polyurethane lacquer (MiCoat™). While the lacquer was still wet, microspheres were sprinkled onto the surface. The purpose of this step was to fix the spheres in place for easier identification and location. The resulting slides were allowed to cure for 7 days. Sample cells were constructed around the polyurethane bound spheres by laying a bead of epoxy (Devcon 2-Ton™) thickened with nano-particulate hydroxy apatite (HA) approximately 5 parts epoxy to 1 part HA) around the edge of the slide. While the epoxy was still sticky, 27 guage needles were laid into the resin so that their tips were within the sample cell. A coverglass was used to seal the top of the cell (figure 3.4).

Swelling measurements. After injecting a phosphate/citrate pH buffer into the sample cell, images of individual microspheres were acquired with a Nikon Labphot™ microscope equipped with a Sony CCD video camera (Model XC-77) and an Apple LCII computer. Images were processed using an Apple LCII and Image 1.44 software.[1] The total area of the projected cross-section of the sphere was measured by counting the number of pixels in the digitized image. Results of these measurements were processed by converting the measured areas into volumes and computing the ratio of the volume at time (t) to the initial volume. Results are converted to % vol swelling (Q) by the following equation

$$Q = \left(\frac{A_t^{3/2}}{A_0^{3/2}} - 1\right) \times 100 \tag{3.3}$$

---

[1] Public domain software available from NIH

where

A_t = area measured at time t, and

A_0 = initial area immediately after injecting buffer solution

The precision of the technique was determined through the use of multiple focus/de-focus cycles on a single object. A micro sphere 300 microns in diameter was focused on. The operator upset the focus and then refocused a total of five times acquiring an image at each repetition. This process was repeated for two more spheres.

During the experimental measurements, five images were acquired at each sampling time, refocusing between image acquisitions. Acquisitions were made just after injection of the pH buffer, then every 15 minutes for 2 hours and once more when swelling equilibrium was reached. A total of 10 acquisition time periods were scheduled for each experiment. The spheres were studied in four different pH environments, namely, 4.5, 5.0, 6.0, and 7.0 phosphate citrate buffers. A total of 1000 data points were collected during the study. The buffer in the sample cell was replaced with fresh buffer solution at least three times during the period of the experiment. The magnification in all images was 100X.

Microsphere Swelling Measurement by Pepper's Technique

Dr. Jun Yan kindly supplied the data from his application of K. W. Pepper's technique to the same microspheres used in the digital swelling measurements described above. Some of his experiments were repeated by the author for confirmation of data and found to be completely accurate.

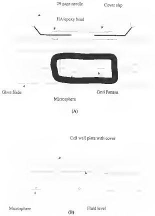


Figure 3 4 Chambers for measurement of microsphere swelling Microscope slide preparation (A) and cell well preparation (B)

Weight gain (due to solvent uptake) was measured by placing approximately 0.5 g of microspheres into the same buffers as those used in the digital image measurements until equilibrium swelling was reached. Swollen microspheres were placed in centrifuge tubes with glass frits at the bottom to eliminate excess liquids from the spheres. After centrifugation, the microspheres were re-weighed and the percent swelling with respect to initial dry weight computed with the following formula.

$$Q_p = \left(\frac{W_f - W_i}{W_i}\right) \times 100 \tag{3.4}$$

where

Q_p = % swelling

W_f = final weight

W = initial (dry) weight

Controls of empty fritted tubes were run at the same time. The effects of retained fluid in the glass frits was eliminated by subtraction of the weight gain of the frits from the micro sphere/frit combination

Modeling of Difference Between Swelling Measurements

Two mathematical models of the potential differences between the swelling measurements were studied. A geometrical model and a physical chemistry model.

Confounding factors Confounding factors that could contribute to the excess weight gain associated with the gravimetric technique include: (1) liquid retained by polymer porosity, (2) defect air bubbles within the polymer, (3) surface adsorption of liquid, and (4) liquid bridge formation between spheres

Geometric model Assume that any changes around one single sphere caused by the known confounding factors can represent the ensemble of microspheres of various sizes We can propose the following model

$$Q_e = \frac{V_p + V_d(Q+1) + \frac{4}{3}\pi[(r+a)^3 - r^3] + 12\pi[r^2 - (r-b)^2]b - 4\pi b^2(3r-b)}{\frac{4}{3}\pi r^3} \tag{3.5}$$

where

Q_e = excess swelling with respect to that measured by the digital imaging technique

V_p = initial pore volume

V_d = initial bubble defect volume

Q = swelling ratio by eq (1)

r = radius of the swollen sphere

a = thickness of adsorbed buffer on the sphere, and

2b = linear length of the meniscus between spheres

To simplify calculations, the meniscus stretching between spheres was assumed to be flat as opposed to the normally hyperbolic shape

The number of liquid bridges around a sphere depends on the packing of the spheres after centrifugation. To keep the number of unknown variables low, the model in equation 3 assumes hexagonal close packing with a coordination number of 12 If a simple cubic cell is assumed, the coefficients should be 6 and 2 for the last two terms of the equation.

Determination of unknown variables. There are five unknown variables in equation 3.5, namely the packing density, V_p, V_ρ, a, and b. V_p was measured by conducting BET analysis with a QuantaChrome nitrogen Autosorb™ (QuantaChrome Corp., Ft. Lauderdale, FL), using approximately 3 grams of microspheres. V_ρ was determined by determining the true density of approximately 0.5 g. microspheres with a nitrogen micropycnometer (Micropyc™ 40, QuantaChrome Corp. Ft. Lauderdale, FL). Packing density could be inferred by comparing true and bulk density, and so bulk density was determined by placing dry microspheres in a pre-weighed 10 cc volumetric flask and forcing the spheres to settle by placing the flask in a shaker bath for 1 week. Spheres lying above the marker line in the flask were poured off and the bulk density was determined from the weight of the remaining spheres. Packing efficiency could be inferred by determining the amount of empty space in the tightly packed spheres.

The remaining variables a and b were determined for each pH environment by fitting the values Q_s and respective radii of the microspheres into equation 3.5 using a curve-fitting program written in Basic.

Physical chemistry model. The volume of the liquid bridge can also be calculated by employing the reduced neck radius, R_2, which is defined as the neck radius, r_2, divided by the radius of the sphere, r. The following equation, based on the works of Kousaka[70], represents the volume of one-half of a liquid bridge with respect to the volume of a sphere:

$$Q_s = \frac{3k_p R_2^4}{16} \tag{3.6}$$

where

k_p = coordination number of spheres

R_2 = reduced neck radius

The neck radius r_2 was determined for each pH by fitting Q_s and respective micro sphere radii in a split-difference algorithm curve fitting program written in Basic.

Measurement of the Swelling of Drug Loaded Microspheres

The kinetic swelling of drug loaded microspheres was measured by digital light microscopy in order to determine whether the mechanism of release of drug was swelling controlled. Travis Arola kindly lent his help in performing these experiments.

Due to the nature of the observed release, only chlorhexidine digluconate, diacetate, and dihydrochloride microspheres were studied in this way. Sets of five of each type of microsphere were studied in pH 4.5, 5.5, and 6.5 citric acid buffers, with a controlled ionic strength of 0.3 M. The measurements were done as described above except that the cell well (96 well polystyrene plates, Corning Glass, Corning, NY) preparation described in figure 3.4 were used instead of the microscope slide preparation. An inverted microscope (Nikon, Tokyo Japan) was used to acquire the images. The final difference was that only one image was acquired per time point, as apposed to the standard five images described above.

Images were acquired at a minimum of 0 1 4, 6 24 48 and 96 hours, with additional time points being added at the discretion of the operator Results are reported as swelling coefficients determined through equation 3 3

Controlled-Release Studies

Two separate controlled-release studies were conducted One was a study of the controlled release of fluoride from microspheres and microparticles of various compositions as a function of pH The second study was a cross-comparison of release rates of tetracycline hydrochloride, chlorhexidine digluconate, chlorhexidine diacetate, and chlorhexidine dihydrochloride all loaded into the same type of micro sphere as a function of pH By studying the release rates in this way, it will be possible to do an analysis of release rate based on polymer type as well as drug type at various pH values

Table 3.3 Citric acid buffer recepies.

Buffer pH	Citric Acid (g)	NaOH (g)	NaCl (g)	Water (ml)	Formalin (ml
4 5	9 607	3 549	12 462	1000	1
5 5	9 607	4 972	8 494	1000	1
6 5	9 607	5 832	3 931	1000	1

Controlled-Release of Fluoride

Four types of microspheres/particles were examined in this study their compositions are listed in table 3.2 Each micro sphere type was examined in pH 4.5 ± 0 04 pH 5 5 ± 0 04 and pH 6 5±0 04 citric acid buffers with an ionic strength of 0 3±0 05

M at room temperature (18.0-22.0 °C). Buffer recipes are listed in table 3.3. Buffers were made by blending pH 4.0 and pH 6.9 buffers while watching a pH meter until the appropriate pH was achieved. The ionic strength was confirmed by using a µOsmette® osmometer (Precision systems, Natick MA). In this way, very precise buffers were attained.

Microspheres and microparticles were sieved to select particles smaller than 150 µm. Between 12 and 20 mg of microspheres or macroparticles were added to each of 3 polypropylene graduated centrifuge tubes (Fisher, plug seal) per pH value studied. Between 13.5 and 15.5 ml of buffer was then added to each tube, as measured by the graduation marks on the sides of the centrifuge tubes. Samples were rotated on a Labquake® sample rotator (Barnstead Thermolyne, Dubuque IA) for the remainder of the study. At the appropriate time, samples of fluid were extracted by drawing all of the fluid out using a sipper tube equipped with a 70 µm mesh attached to the opening (to prevent extraction of the particles), see figure 3.5 b. The fluid was replaced each time with fresh buffer.

Sample preparation. All of the sample fluid from each tube was poured into a polypropylene graduated centrifuge tube (Fisher, plug seal). The amount of fluid added was recorded and the cap removed from the tube. Uncapped extract tubes were dried in a large vacuum oven equipped with an internal fan, and with the door slightly open to allow vapors to escape. The temperature of the drying oven was approximately 80° C, drying time varied from 3 to 10 days.

Dried samples were reconstituted in one of two ways. Those samples that were comprised of dried pH 4.5 buffer were reconstituted with 1.0 mL of 0.6 N NaOH (Fisher,

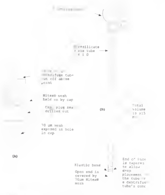


Figure 3 5 Microsieves made from polypropylene centrifuge tubes and 70 µm Nitex® mesh (A) Sipper tube made from borosilicate glass tubing, a rubber band, and 70 µm Nitex® mesh (B) lot 712926)(to adjust the pH) and 1.0 ml of triple ionic strength adjustment buffer (TISAB) (Fisher, Atlanta GA). The remaining samples were reconstituted with 2.0 ml of TISAB. All reconstituting fluids were measured accurately using an adjustable pipetter (Oxford, Fisher, Atlanta GA).

Sample measurement. Fluoride content in samples was measured using an ion-selective electrode (Accumet, Fisher, Atlanta, GA). A calibration curve, based on water/KF standards mixed 1:1 with triple ionic strength adjustment buffer (TISAB) was made by graphing the log of the concentration against the difference between the voltage at 0 ppm and the standard being measured. A total of 8 standards were used to produce a linear calibration from 0.5 ppm to 1000 ppm. A least squares fit (in Sigma Plot 4.0, Jandel Corporation, 1996, USA) was used to determine the slope and intercept of the standard curve. The calibration was checked every 2 hours and a separate calibration curve was made every 6 hours or less. The number of mV measured for each sample was subtracted from the number of mV measured in a 0 ppm standard. This difference was then converted into the number of mg. released using the following formula

$$R = \frac{10^{(m\cdot\Delta)+b}}{500} \qquad (3.7)$$

where

R = mg KF released

m = slope of calibration curve

b = intercept of calibration curve

Data analysis. The cumulative release was computed from this data by summing the number of mg released over time and dividing by the percent loading multiplied by the number of mg of microspheres *id est*

$$\frac{M_t}{M_\infty}=\sum_{n=1}^{t}\frac{R_n}{W*L} \tag{3.8}$$

where

M_t = Cumulative release at time t

M_∞ = Maximum amount of release expected, usually = L

W = Mass of microspheres used in experiment

L = Fractional loading of microspheres

R_n = Mass of released drug at time n

Data from the controlled-release studies was analyzed by simply plotting M_t/M_∞ versus $t^{1/2}$. Visual observation of these curves indicated Fickian or non-Fickian release behavior. A more complicated analysis by non-linear regression analysis was avoided due to the limited potential of this technique in applications involving initially dry microspheres.

Controlled-Release of Antibiotics

The methods used in studying the controlled-release of chlorhexidine salts and tetracycline were very similar to those already described for the fluoride case. The major differences lie in the analysis of the samples.

Sample preparation. Microspheres loaded with chlorhexidine digluconate, diacetate, and dihydrochloride and tetracycline hydrochloride were prepared and sieved as previously described. The microspheres were weighed into tubes, buffers were added, and the tubes were allowed to rotate as previously mentioned. Control samples comprised of 1 ml of 20-30 ppm of each drug in a water standard, were added to 13 ml of each buffer type at the beginning of the study. These control samples were placed onto the sample rotator next to the experimental samples. The rotator was placed into a dark cabinet (the drugs have been reported to degrade under UV light) and maintained at room temperature during the duration of the experiment. At 6 hours, 24 hours, 3 days, 8 days, and 30 days samples were extracted and the buffer was replaced as previously described. Unlike in the fluoride study case, the samples were analyzed without further modification.

Sample measurement. Approximately 2 ml of each sample was placed into a semi-micro acrylic cuvette (Fisher Scientific, USA) and analyzed by UV spectrophotometry (Spectronics Genesys, Spectronics Inc., Rochester NY). Separate water-based standards were used for each drug type. Water standards and blank solutions were used because no differences were found between standards made with buffers and those made with water. No pH dependance was found in the spectrum of any of the drugs. Analysis of chlorhexidine samples was done at 256 nm. Tetracycline samples were analyzed at 358 nm.

Data analysis. Release data was analyzed as described previously, using formula 8. Plots were made of each data set against linear time. These plots were then examined visually to determine rough trends in release as a function of pH and polymer type. Further analysis of the kinetics of release was then done.

The six-hour time point represented, for the most part burst release data, which is not indicative of the later release behavior. For this reason, a database was prepared in which the six-hour data had been subtracted from all subsequent values. A linear regression of this data against a time to the ½ power axis was then used to determine if the release followed Fickian kinetics. The r^2 values were used to determine the degree of linear fit. Any r^2 value below 0.95 indicated non-Fickian release.

Selected non-Fickian data was then plotted against linear time. The r^2 values for these regressions were used to determine if the release was zero-order. Again, any r^2 value below 0.95 indicated non-zero-order release.

Efficacy of Microspheres in Broth *S. mutans* Culture

The ability of microspheres to kill bacteria in a pH controlled manner was tested in a broth *S. mutans* (NG-8, provided by Elaine Beem of Oral Microbiology) culture. A modified tryptic soy broth was prepared according to the standard recipe (Difco, Fisher Scientific, Atlanta GA) except that the buffering compound (sodium phosphate, dibasic) was left out. This ensured that the broth had a low buffering capacity that would allow the microbes to naturally bring the pH down through the production of acid.

A 20 ml tube of broth was inoculated with *S. mutans* using a cotton swab, and allowed to grow overnight in a dry incubator at 35.5° C, enclosed in a 1 gallon Ziplock®

bag with a BBL gas pack (Fisher Scientific, Atlanta GA). This broth was then used to inoculate 250 ml of sterile broth to be used as a stock inoculant.

Chlorhexidine digluconate and chlorhexidine diacetate loaded microspheres were studied in this experiment. Approximately 60 mg of microspheres were added to sterile glass culture tubes. The tubes were filled with sterile broth and held overnight to remove surface drug from the microspheres; this broth was decanted off. 15 ml of the inoculated stock broth was then added to each tube. Three tubes for each microsphere type were prepared, as well as three control tubes without microspheres. Tubes were incubated in anaerobic bags in a 35.5° C incubator as described above. The bacterial concentration (colony forming units/ml, or CFU/ml) and the pH were determined at 0, 4, 24, and 48 hours.

The bacterial concentration in each tube was determined by preparing dilutions of the broth in sterile water. Sterile water was measured out in a sterile graduated cylinder and poured into a sterile 120 ml vial. Inoculated broth was measured using a sterilized micropipet. 100 µl of broth was added to 100 ml of sterile water to produce a 1:1x10^3 dilution. This dilution was then serially diluted to produce 1:1x10^5 and 1:1x10^6 dilutions in sterile water. 100 µl of each dilution was then swirl plated onto standard tryptic soy agar plates (Difco, Fisher Scientific, Atlanta, GA) using a sterilized glass or plastic L-shaped swirling tool. These plates were then appropriately marked and allowed to grow 24 hours at 35.5° C in the anaerobic bag described above. Colonies were counted on each plate and multiplied by the dilution factor (number of ml plated times the dilution factor of the sterile water/broth mix).

The pH was measured using a pH probe which was sterilized in bleach ethanol solution and then rinsed in sterile water

Graphs of the bacterial concentration versus pH were prepared and studied to determine if the microspheres had any effect on bacterial growth and survival.

CHAPTER 4
RESULTS AND DISCUSSION

Introduction

A large amount of attention was paid in Chapter 3 to the synthesis and preparation of the micro spheres and microparticles examined in this dissertation. Since the techniques involved with producing unloaded microspheres have been well studied by other investigators, and are not the crux of this document, they will not be discussed in the results and discussion section.

Below, the results of synthesis experiments for intrinsically loaded microspheres and microparticles, and the choice of loading techniques, the physical characterization of the particles studied, as well as their release, swelling and biological efficacy will be described in detail.

Synthesis of Fluoride Loaded Microspheres and Microparticles

Attempts were made at synthesizing both microspheres and microparticles loaded with fluoride. Only the loading of microparticles was successful.

Fluoride Loaded Microspheres

The yield of microspheres during these experiments, and the quality and size of the microspheres obtained was comparable to that experienced without the presence of fluoride salts It was found that potassium fluoride flocculated out of the monomer solution just before it gelled, even with vigorous stirring This could be caused by the temperature exceeding the upper critical solution temp of KF A more likely explanation is that the increased hydrophobicity of the solvent caused a reduced compatibility with the KF and lead to rapid flocculation. Sodium fluoride suffered this problem, but not to as significant an extent as KF For this reason, sodium fluoride was used in subsequent experiments

In experiments with sodium fluoride, it was found that no appreciable (no crystals observable in the matrix) fluoride loading occurred, even when the aqueous phase was saturated with sodium fluoride A likely explanation is that the sodium fluoride particles segregated to the surface of the forming microspheres, due to hydrophobic/hydrophilic interactions, and either migrated into the aqueous phase, or were washed off during subsequent processing

Fluoride Loaded Microparticles

The fluoride loaded microparticles were successfully produced using potassium fluoride While the flocculation problem continued in these syntheses, it was found that after a critical viscosity was reached, the fluoride re-suspended into the monomer/polymer solution The yield was 100% After xylene processing, approximately 30% of the particles were below 150 μm in size. With more effort, the remaining 70% could have easily been rendered smaller than 150 μm.

Loading Techniques

As mentioned previously, the methods used to load the microspheres were an integral part of the study. Although past researchers have used the solvent (methanol) employed here, it was not at all apparent from the start that this was the ideal choice. Secondly, the choice of drugs for loading into the microspheres was not a simple one. Many of the potential choices were ruled out due to solvent incompatibility or other chemical factors (such as degradation during the loading process).

Since the polymer/solvent compatibility was relatively well known, thanks to the work of Dr.'s Yan, and Toreki, the major tasks were:

(1) Find a drug that is soluble in a compatible solvent.

(2) Optimize the loading percentage by optimizing the swelling and drug concentration in the solvent concomitantly.

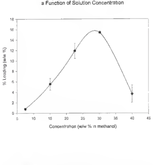
(3) If necessary, optimize the drying and end-processing steps to maximize loading.

Drug Solubility

According to the methods mentioned in Chapter 3, the solubility of several varieties of chlorhexidine was measured in a variety of solvents, all of which swell the polymers studied here. Fluoride was studied in the same way. It was found that chlorhexidine, un-derivatized was not appreciably soluble in any of the solvents studied with the exception of acetic acid (in which chlorhexidine was changed to chlorhexidine diacetate) In a similar manner, sodium fluoride was not appreciably soluble in any solvent, reaching its highest solubility at 2% by weight in water Salts of chlorhexidine, and potassium fluoride however were found to be highly soluble in methanol as well as some tetrahydrofuran (THF)/water solutions Since previous work had been done using methanol as the loading solvent, this was chosen, since it was no worse or better than the THF/water solutions.

Effect of Drug Solution Concentration on Loading

Percentage loading as a function of drug concentration in solution was studied for the chlorhexidine digluconate case Due to the extremely high solubility of this drug in methanol, a maximum in the loading was observed at a point below the maximum solubility of drug in solution. The results of the experiment are displayed in figure 4 1 A maximum of around 16% by weight loading was observed when the concentration of drug in solution was about 30% by weight A sharp drop in loading can be observed near the maximum solubility of the drug at 40% This phenomenon could be attributed to one of two possible sources (1) The swelling of the polymer in this concentrated solution is decreased relative to the swelling at 30% drug concentration. (2) The swelling is the same but the ionic strength of the drug solution prevents migration of the drug into the polymer matrix.



Loading of Chlorhexidine Digluconate as a Function of Solution Concentration

Figure 4.1: Percent loading of chlorhexidine digluconate in microspheres as a function of drug concentration in methanol solution. The solid line is a computer generated spline curve fit, and is not necessarily representative of any real phenomena.

To confirm or eliminate option (1), an attempt to measure the swelling of these microspheres was made. Significant problems with drug crystallization due to slight evaporation, combined with the large difference in refractive index between different concentrations of solution prevented the development of any meaningful data with regard to swelling in this system.

The swelling of microspheres in potassium fluoride solutions was also studied. In these microspheres there was no maximum in swelling observed, and the calculated percent loading, as determined by multiplying the fractional swelling by the fractional concentration of drug in solution, was found to have a maximum at the maximum solubility of KF in methanol (12%). The results of this study are displayed in figure 4.2. Correlating data for percent loading at each of these concentrations was not obtained.

Drying Step

As was mentioned, early attempts at loading KF into microspheres yielded very poor results (loading ~0.005%). In these early samples, all drying was done at room temperature, under vacuum. A study was undertaken to see if the cause of this low loading was due to the migration of KF to the surface of the microspheres during the slow drying process and the subsequent removal of the drug by washing.

As stated, xylene was used in pre-loaded, but wet, microspheres to try to force the KF out of solution inside of the microspheres. This was intended to prevent the KF from

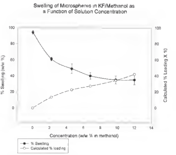


Figure 4.2 Swelling ratio of microspheres in KF/methanol solutions as a function of KF concentration

being able to diffuse to the surface, since it would be in solid form once the xylene reached sufficient concentration in the interior of the micro sphere.

A second attempt involved hard-freezing the microspheres in liquid nitrogen and rapidly lyophilizing them. In this way, micro crystals of KF would form on the inside of the microspheres. Once dry, the microspheres were annealed above their dry T_g to force collapse of the freeze-dried gel. The micro crystals of KF remain trapped in the collapsed polymer network after annealing.

Both these attempts yielded some loading of KF into the microspheres. The xylene loaded microspheres in this study contained 0.028±.014% w/w KF. The freeze-dried microspheres contained 0.36±.052% w/w KF. The measured loading rate on the second set of microspheres is obviously higher. This difference was statistically very significant, even by a very stringent 2-sigma test. For this reason, all KF loaded microspheres were loaded by lyophilizing and annealing.

The difference seen between these two results is unexpected. Xylene is a very poor solvent for KF. One would expect it would have caused rapid precipitation inside of the microspheres. One likely explanation is that the KF diffused rapidly enough from the microspheres that it traveled with the methanol, and was not left behind as a precipitate. A second explanation is that the lyophilized microspheres simply had more KF that was extractable under the experimental conditions. This second possibility would be particularly believable if there was incomplete collapse of the gel structure during annealing. This latter explanation is unlikely since, in the presence of methanol (that was used to extract KF during percent loading analysis), the gel structure of the lyophilized microspheres will rapidly become similar to that of the xylene loaded ones

Particle Characterization

Both microspheres and microparticles were characterized in the same manner. Work done by other researchers has highlighted the solubility and pH dependant behavior of this polymer system [2, 28-29, 31-33, 35-37, 38, 64]. Particle characterization in this study was limited to size distribution determination and percent loading analysis. Thermal analysis was not conducted since the state of interest in these microspheres is that in which they exist as gels, which means, by definition, that the T_g lies at or below the temperature of the experiment.[16]

Particle Size Determination

Particle sizes were measured as described in chapter 3. The relevant results are given in table 4.1. The mean radius **<R$_n$>** was computed as a normal mean. The weighted mean radius <R$_w$> and the polydispersity index (PDI) were computed as follows

$$<R_w> = \frac{\sum_i R_i^2}{\sum_i R_i} \qquad (4.1)$$

$$PDI = \frac{<R_w>}{<R_n>} \qquad (4.2)$$

where

R_i = the radius of sphere i

PDI = the polydispersity index, indicating the breadth of the particle size distribution.

The particles all fell roughly within the same size range with the exceptions of the T51 microspheres, which were much smaller and had a large PDI, and the microparticles. The numbers reported for the microparticles are for those which were sieved to elliminate particles larger than 150μm. The PDI of the microparticles was large, as would be expected due to the method of production. A rough attempt was also made at determining the aspect ratio of the microparticles. The aspect ratio ranged from 1.0 to 2.0, with the mean lying closer to 2.0. A large enough sample size was not available conduct an accurate and detailed analysis.

Table 4.1: Size and size distribution of microspheres and microparticles in microns

Sample	$\langle R_n \rangle$	σ	$\langle R_w \rangle$	PDI	n	minimum	maximum
T4	151	48	165	1.09	26	13	226
T40	113	26	119	1.05	57	40	160
T51	61	29	73.7	1.21	59	13	107
particles	104	62	139	1.34	26	13	253

Percent Loading Analysis

The percent loading analysis for each type of microsphere was done as described in chapter 3. The relevant results are depicted in table 4.2.

The loading efficiency was calculated by dividing the percent loading by the concentration of drug in the loading solution and multiplying by 100. This should give a rough indication of the drug/polymer compatibility and diffusion rates of the drug in the polymer matrix

Both the highest loading efficiency and highest absolute loading were observed with chlorhexidine dihydrochloride. The lowest efficiency was seen with tetracycline HCl. The tetracycline HCl results are difficult to believe, however, since the UV/Vis spectrograph at the time of measurement indicated that the drug had degraded in solution during the time of the experiment. Further attempts, using other solvents, including methanol, resulted in the same behavior. The microspheres are visibly very dark in color, indicating that the loading is probably much higher than this data would seem to indicate.

Table 4.2: Percent loading analysis				
Batch Number	Particle Type	Drug Type	%Loading	Loading efficiency (%)
JWF062B	T40	chlorhexidine digluconate	1 19± 08	5 2
JWG022A	T40	chlorhexidine diacetate	2.64± 69	11 5
JWG017A	T40	chlorhexidine dihydrochloride	7 30± 6	60 8
JWG013A	T41	tetracycline hydrochloride	180± 004	1 8
JWF074A	T4	KF	453± 131	4 3
JWF074B	T40	KF	1.21± 13	11 4
JWF074C	T51	KF	850± 236	8 0
JWF090A	micro-particles	KF	5 88± 05	N/A

The discrepancy between loading efficiencies of chlorhexidine HCl and the digluconate and diacetate derivatives could be due to a number of factors.

(1) The lower concentration and thus the lower ionic strength of the solution used with chlorhexidine HCl (due to its lower solubility in methanol)

(2) Different diffusion rates due to molecular size/weight

(3) Chemical interactions between the ligand molecule (HCl) and the polymer matrix.

While the possibility of an ionic strength factor taking effect exists it is unlikely in light of the data on solution concentration versus percent loading described earlier

The difference in molecular weight and size between these compounds is significant. The ligand is between 6.5 and 44% of the total weight of the compound for chlorhexidine dihydrochloride and digluconate respectively This means that there is a 36.5% difference in the weight of these species. This is may be enough to explain the disparity in the observed loading However chemical interactions seem a more likely source

Chemical factors could be due to a number of phenomena

(1) Steric hindrance between the ligand and the polymer mesh

(2) Local chemical environments due to the ligand which increase or decrease the polymer mesh size during loading and diffusion.

While steric hindrance cannot be ruled out easily, the argument used for diffusion differences above, will still hold fairly well This leaves only the interaction of the ligands with the polymer on a local basis as the source of the disparity

Since acidic species are present as ligands on both chlorhexidine dihydrochloride and diacetate it makes sense that the drug is changing the global or local pH to some degree which forces the polymer matrix to become more open to the influx of drug during loading. The lower pK_a of HCl (-7) relative to that of acetate (4.76)[71] would serve to indicate that the polymer is being swelled to a greater extent during loading in the former case. Since glucose is a relatively neutral molecule, it does not have this effect upon the polymer matrix, and so has a low loading efficiency.

The possibility remains that the drugs were released from the polymer at drastically different rates, and that the microspheres were not at equilibrium when the samples were tested for percent loading. Considering the controlled-release data to be presented later on, this seems an unlikely case, and the data is most likely accurate.

Potassium fluoride enjoyed a relatively good loading efficiency in both T40 and T51 microspheres. The T4 microspheres did not load well with KF. The observed difference could be due to one of the following factors:

(1) The ability of the polymer to bind fluoride and its resulting inability to release fluoride

(2) The hydrophobicity/hydrophilicity of the polymer matrix

The first point above can be ruled out immediately due to the fact that any trend it would show would run contrary to that seen in the data. The compositions that contain a higher amine content are also the ones that load with fluoride better, contrary to what this scenario would predict. The second scenario is supported by the data, however. Of the microspheres loaded, composition T4 had the highest content of a hydrophobic species and also the lowest loading rate. While the hydrophobic monomer content of the T40 and T51 compositions are similar, T40 showed a higher loading efficiency (11.4% relative to

8.4%, respectively). This can be explained by the fact that the hydrophobic species in T40 is methyl methacrylate instead of the slightly more hydrophobic ethyl methacrylate in T51.[32]

Whether these microspheres would exhibit the reverse loading trends with a hydrophobic drug like chlorhexidine remains to be tested. It is unlikely that this will occur, however, since concomitantly with higher hydrophobic content, we usually observe a lower swelling in methanol.

Measurement of the Swelling of Microspheres

Polymer swelling can be directly correlated with release rates when there is no drug/polymer interaction.[28, 29, 34, 37] As a result, swelling in both loaded and unloaded microspheres was measured. The results of studies into the high precision method of digital light microscopic measurement of swelling and its comparison to a standard technique will be discussed first. The application of this technique to drug loaded microspheres will be described second.

High-Precision Measurement of the Swelling of Microspheres[69]

As described in chapter 3, microsphere swelling was measured by two techniques. The precision of the digital image acquisition technique was determined. Then the digital technique was compared to the classical method of Pepper.[68] Modeling of the difference between the two techniques was done to prove that the difference was due to fluid retention due to absorption on the microspheres.

Precision The measured precision of the microscopy technique was high. The standard deviation of the volume of an individual sphere, as a percent of the mean, ranged from 0.10 to 0.50% with a mean of 0.26%. If a 2σ rule is adopted, then the precision of this technique is better than 0.52% of total swelling.

Comparison of two techniques Table 4.3 shows the percent swelling increase at equilibrium by the digital light microscopy technique and the Pepper technique. The results have validated the concern that gravimetric methods will yield excess swelling. It is surprising that the excess value actually constitutes as much as 84% of the total weight gain in some cases.

Table 4.3 Equilibrium swelling values for microspheres at various pH's as measured by digital imaging technique and Pepper's technique

pH	Q_a	Q	Q_w	Q_e	$(Q_w-Q)/Q_w x100$
4.5	84.5	77.7	217.5	139.8	64.3
5.0	20.6	9.4	59.0	49.6	84.1
6.0	18.0	8.8	36.6	27.8	76.0
7.0	11.7	7.5	33.5	26.0	77.0

Q_a = Average area gain (%) by digital imaging technique
Q – Average volume gain (%) by digital image technique
Q_w = Average weight gain (%) by gravimetric technique
Q_e = Average excess swelling (%)

Nitrogen BET interpretation did not reveal the presence of any significant internal porosity within the microspheres. Additionally the true density of the microspheres as measured by nitrogen micropycnometry exactly matched the theoretically predicted density of 1.19 g/cc. Therefore it is unlikely there were significant voids within the spheres. For the present polymeric system then, the null values of porosity (V_p) and bubble volume (V_b) led to the elimination of the first two terms in the model proposed in equation 3.5. Initial runs of a two parameter model involving both a and b indicated that the value of a was of the order 10^{-7} µm. A null value for a was assumed. True and bulk density measurements also show that the packing efficiency of the microspheres in this study was 38%, close to the 43% seen in simple cubic (SC) cells with spheres all of the same size. This indicates that the actual packing of microspheres used in the gravimetric technique would be close to a simple cubic cell after centrifugation. The proposed model (equation 3.5) can then be reduced as follows:

$$Q_e = \frac{\kappa_p b^2(3r-2b)}{4r^3} \tag{4.3}$$

Finally the value of the meniscus length b, was determined using the radius and excess volume calculated for each condition. The parameter κ_p was posited at 6, assuming simple cubic packing. Results are shown in table 4.4.

Table 4.4 The values of b calculated for different coordination numbers in various pH buffers

pH	Average Radius r (µm)	Average Swelling (% volume)	b (HCP) (µm)	b (SC) (µm)
4.5	70.9	77.7	33.8	59.6
5.0	60.3	9.4	15.6	23.2
6.0	60.2	8.8	11.3	16.5
7.0	59.9	7.5	10.9	15.9

Based on the values of **h**, which was the half length of the assumed flat meniscus, the reduced neck radius can be calculated by the following equation

$$R_2 = \frac{\sqrt{r^2-(r-h)^2}}{r} \tag{4.4}$$

The value of $\mathbf{R_2}$ was then used to calculate $\mathbf{Q_e}$ by equation 3.6. Results for the simple cubic case are given in table 4.5. A nearly perfect match of the swelling difference computed with equations 3.3 and 3.4 was observed.

Table 4.5 The values obtained for the excess volume equations 3.5 and 3.6 are similar

pH	Radius r (µm)	h (µm)	R_2	Q_e (%)	Q_e (Eq. 3.6) (%)
4.5	70.9	59.6	0.99	140	107
5.0	60.3	23.2	0.79	50	44
6.0	60.2	16.5	0.69	28	25
7.0	59.9	15.6	0.68	26	24

Discussion of models. As can be seen from the data presented, a nearly perfect match between the models is achieved. The difference between the two models is greatest at high degrees of swelling (140% actual swelling vs. 107% as shown by equation 3.5). Presumably this is where the assumption of a flat meniscus in equation 3.5 breaks down.

Each of these models for liquid bridge formation were derived from separate approaches. The model in equation 3.5 was derived from a purely geometrical standpoint. The model distilled from the works of Kousaka[70] in equation 3.6 were derived from physical chemistry arguments. Their equivalence is not exact, as has been shown in the modeling data. This is to be expected, because a brief mathematical analysis shows that the two models are only equivalent when r_2 is equivalent to **(4/3)r**--a physical impossibility. Each model, however, does aid in the visualization of the phenomenon of excess fluid retention during swelling experiments.

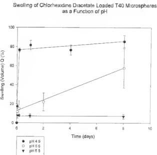
The results of these modeling experiments serve to prove that the gravimetric methods used to measure swelling in microspheres will be adequate at high degrees of swelling, since the percentage error due to liquid bridge formation is low in these cases. In the case of low degrees of swelling, however, the gravimetric techniques are inadequate, leading to as much as an 84% over-estimate of the actual swelling of the micro sphere.

Measurement of the Swelling of Drug Loaded Microspheres

The kinetic swelling of chlorhexadine diacetate, digluconate, and dihydrochloride microspheres was measured with the digital light microscopy technique. Figures 4.3 through 4.5 show the results of this work.

From the standard deviations seen in the data shown, it is obvious that the elimination of the 5 focus/refocus cycles per measurement was detrimental to the technique's sensitivity. However, the general trends in the data are readily ascertained from the graphs displayed.

Chlorhexidine diacetate (figure 4.3) appears to swell according to Fickian diffusion at pH 4.5. Equilibrium swelling at this pH is reached within 5 hours. At pH 5.5, the data



Swelling of Chlorhexidine Diacetate Loaded T40 Microspheres as a Function of pH

Figure 4.3 Swelling of chlorhexidine diacetate loaded microspheres as a function of pH

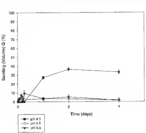


Figure 4 4: Swelling of chlorhexidine digluconate loaded microspheres as a function of pH

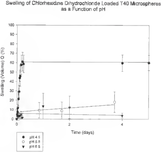
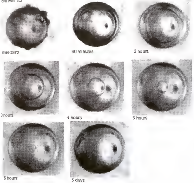


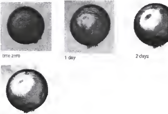
Figure 4.5: Swelling of chlorhexidine dihydrochloride loaded microspheres as a function of pH



Chlorohexidine Dihydrochloride loaded microsphere
test started 7/3/97
pH=4.5 I=0.3 citric acid buffer
cell well A2

Figure 4.6: Swelling fronts were commonly observed in these microspheres

chlorohexidine Digiucinate loaded microsphere
Test started 7/11/97
pH~4 5 I~0 3
Cell well A3



time zero

1 day

2 days

4 days

Figure 4.7: The chlorhexidine diacetate and digluconate loaded microspheres frequently showed a wrinkled surface when swelling began.

is scattered but appears to have a linear character At pH 6 5 the swelling is 0 for the length of the study

Chlorhexidine digluconate (figure 4 4) displays Fickian behavior at pH 4 5 Equilibrium swelling at this pH is reached within 50 hours There appears to be a lag phase to the swelling curve, with the swelling being virtually zero until a sudden increase in rate between 6 and 24 hours The swelling at pH 5.5 and 6 5 remained near 0

Chlorhexidine dihydrochloride (figure 4 5) also showed Fickian swelling behavior at pH 4 5 The equilibrium time at this pH was approximately 6 hours. The swelling appears to be the same at pH 5 5 and 6 5, lying somewhere around 10% The standard deviations make more detailed analysis of this data difficult to justify

Figure 4 6 shows a typical swelling front as observed with all of these microspheres at pH 4.5 At other pH's the swelling front was not as easy to observe, but in those microspheres that did swell, it was present.

The chlorhexidine diacetate and digluconate microspheres showed a wrinkled surface morphology that made observation of the swelling front difficult to observe. As can be seen in figure 4 7, by 2 days, the wrinkled morphology is gone When the wrinkled morphology is seen It is probably due to a shallow layer of gel that has formed on the surface expanding to a size larger than the underlying un-swelled sphere Since the glassy polymer forces this gel layer to maintain its radius, the gel wrinkles At later times the strain exerted by the core is dissipated through a thick gel layer eliminating wrinkling The reason that this behavior is not seen in the chlorhexidine dihydrochloride microspheres is either that swelling is so rapid, the gel layer on the surface is never thin enough to be observed, or that the transition between gel and glass is more gradual, allowing relaxation of the stresses over a larger volume.

Controlled-Release Studies

Controlled-release of fluoride and antibiotics including chlorhexidine and tetracycline was done in two separate experiments. In each study, the release at pH 4.5, 5.5, and 6.5 was measured as a function of time. The results from each of these studies will be discussed in the order mentioned above.

The general trends in the data from these studies will be briefly described. Following this general presentation of the controlled-release data, attention will be paid to the controlling factors in the release.

Fluoride Controlled-Release

The release profiles for T4, T40, and T51 copolymers and microparticles at pH 4.5, 5.5, and 6.5 were plotted in two different ways. First, the data was plotted as cumulative release (M_t/M_∞), using the M_∞ derived from percent loading analysis, against a linear time axis. Second, the data, with the point at 6 hours subtracted from the cumulative release (since this represented the burst effect at the beginning of release) was plotted as M_t/M_∞ against a $t^{1/2}$ axis. Each curve will be presented and described in turn. A general discussion of the trends and proposed meaning of the data will be reserved for late in the discussion.

Linear time plots of data. Figure 4 8 shows a plot of the release data for T4 microspheres As can be readily seen from the graph, the release does ont follow the pattern expected There is very little release of KF at low pH, and apparently a higher release at pH 6 5

Figure 4 9 show the kinetics of release of T40 microspheres Here the trend of release is exactly the opposite of that seen previously Here, release occurs most rapidly at pH 4 5 and as expected there is slower release at pH 6 5 What should be noted is that while the release rate is reduced at pH 6.5, it is not stopped Also there seems to be a smooth transition point for release onset That is, the release of KF at pH 5 5 is intermediate between that at pH 4 5 and pH 6 5

Figure 4 10 displays the release of KF from T51 microspheres over time Here again as with the case of T40 microspheres, the expected trend in release behavior relative to pH is observed The most rapid release is at pH 4.5, while there is little release at pH 5.5, and 6 5 There is no difference between the release at pH 5.5 and that at 6 5 Here, the onset pH seems to be a sudden occurrence between pH 5 5 and 4 5

Finally figure 4 11 shows the release from the microparticles The release in this case is a burst release followed by little or no release at all past the 6 hour time point. The only difference between pH's is the total amount of drug that is released during the burst At pH 4 5 100% of the loaded KF is released almost immediately At pH's 5 5 and 6.5 however only 70% of the drug is released, and there appears to be no continued release

Linear regression analysis against $t^{1/2}$ During the first 6 hours of release many microspheres release a "burst" of loaded drug, and then begin to settle into their

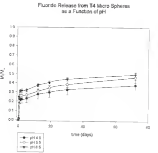


Figure 4.8: Linear time plot of fluoride release from T4 microspheres.

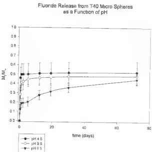


Figure 4.9 Linear time plot of fluoride release by T40 microspheres.

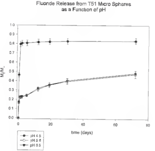


Figure 4.10 Linear time plot of fluoride release by T51 microspheres

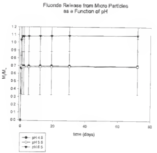


Figure 4.11 Linear time plot of fluoride release by microparticles

Table 4 6 Linear regression analysis of controled release curves versus $t^{1/2}$

Drug	Polym	n	pH	Intercept	Slope	r^2	Fickian ?
KF	T4	7	4 5	0347	0231	9800	Y
KF	T4	7	5 5	0130	0292	9889	Y
KF	T4	7	6 5	0324	0267	9728	Y
KF	T40	7	4 5	0330	0039	9510	Y
KF	T40	7	5.5	1326	0098	8075	N
KF	T40	7	6 5	1 537	0040	9860	Y
KF	T51	7	4 5	3317	0049	8922	N
KF	T51	7	5 5	0159	0361	9792	Y
KF	T51	7	6 5	0136	0370	9767	Y
KF	MP	7	4 5	0212	0006	9352	N
KF	MP	7	5 5	0212	0006	9352	N
KF	MP	7	6.5	0440	0019	9789	Y
Chx digluconate	T40	3	4 5	1000	1820	9984	Y
Chx digluconate	T40	3	5.5	- 0181	0465	9286	N
Chx digluconate	T40	3	6 5	-9 316	0483	9174	N
Chx dihydrochloride	T40	3	4 5	1838	0597	8958	N
Chx dihydrochloride	T40	3	5 5	0822	1329	9684	Y
Chx dihydrochloride	T40	3	6 5	0575	0109	9757	Y
Chx diacetate	T40	3	4.5	2451	1500	9243	N
Chx diacetate	T40	3	5 5	- 2195	2786	9940	N
Chx diacetate	T40	3	6 5	- 0237	0457	9996	Y
Tetracycline HCl	T41	3	4 5	- 0004	0008	9999	Y
Tetracycline HCl	T41	3	5.5	- 0005	0009	9980	Y
Tetracycline HCl	T41	3	6 5	- 0004	0008	9981	Y

*MP=microparticles, n=number of points analyzed Plym=polymer type equilibrium release rates For this reason in the linear regression analysis, this "burst" phase was subtracted from the cumulative release By careful linear regression analysis of the resulting data, the kinetics can be judged "Fickian" or "non-Fickian" A near perfect fit to the linear line of regression indicates Fickian behavior, while anything short of perfection results in a judgement of "anomalus" release Values of the parameters found in the linear regression are depicted in table 4 6

In figure 4 12 a plot of T4 microspheres shows a nearly perfect linear fit for all curves. This indicates that the diffusion in all cases is Fickian in nature All three curves have very close to the same slope

Figure 4 13 shows a plot of T40 microspheres in which a linear fit was found for the curves at pH 4 5 and 6 5, but not for the one at pH 5 5 (r^2=0 8075) This indicates that while the diffusion at pH 4.5 and 6 5 is Fickian that at pH 5 5 is anomalous

Figure 4 14 shows a plot of T51 microspheres in which there is a linear fit for both pH 5 5 and 6 5, but not at pH 4 5 The slope of the curves at pH 5 5 and 6 5 indicates that release is ongoing but slow at these pH s

Contrary to what was expected, there is not a perfect linear fit to the cumulative release data collected from microparticles. As depicted in figure 4 15, however from the very slight slope, one can assume that there is little or no ongoing release The poor r^2 values obtained are probably due to sample error and not due to any real phenomena.

Figure 4 16 shows a linear regression plot against linear time of the KF release data from T40 microspheres at pH 5 5 and T51 microspheres at pH 4 5 As can be seen, neither of these fit this linear model Refer to Table 4 07 for relevant linear regression parameters

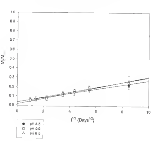


Figure 4.12: Linear regression of modified KF release data for T4 microspheres versus $t^{1/2}$

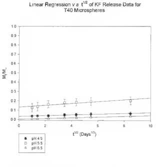

Linear Regression v s $t^{1/2}$ of KF Release Data for T40 Microspheres

Figure 4 13 Linear regression of modified KF release data for T40 microspheres versus $t^{1/2}$ Because the initial burst release has been subtracted from the data, at pH 4 5 release appears to have a lower absolute value than the release at pH 5 5

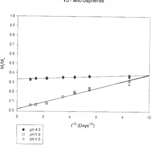


Figure 4.14 Linear regression of modified KF release data for T51 microspheres versus $t^{1/2}$

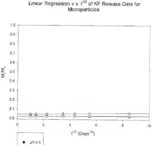


Figure 4 15 Linear regression of modified KF release data for microparticles versus $t^{1/2}$

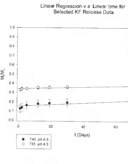


Figure 4.16: Linear regression of modified KF release data for T40 microspheres at pH 5.5 and T51 microspheres at pH 4.5 versus linear time

Comparison of release rates By comparing the previous figures, it is apparent that at pH 4.5 the order of release from least to most during the period of the experiment is T4, T40, microparticles, and T51. At pH 5.5, the order of release from least to most during the period of the experiment is T4, T51, T40, and microparticles. At pH 6.5, the order of release is T40, T51, T4, microparticles.

Antibiotic Controlled Release

As with the fluoride controlled-release data, the antibiotic release data will be plotted in a number of ways. First, graphs of the release of the three different chlorhexidine derivatives (digluconate, dihydrochloride, and diacetate) will be shown as M_t/M_∞ plots versus linear time. The next series of plots will show a linear regression of the data with 6 hour "burst" data subtracted, versus $t^{1/2}$. Analysis of these graphs, to determine the kinetics of release, will be done as in the case for fluoride release. In these graphs, M_∞ is taken as the percent loading value determined as described in Chapter 3. The plots of tetracycline controlled-release will not include an M_t/M_∞ ordinate, since due to drug instability it was impossible to determine actual percent loading.

Linear time plots of data Figure 4.17 shows the pH controlled-release plot of chlorhexidine digluconate from T40 microspheres. As can be seen, there is the expected pH controlled release with the transition to rapid release occurring between pH 4.5 and 5.5.

Figure 4.18 shows the release of chlorhexidine dihydrochloride as a function of pH. Here, while the expected pH dependant release is observed, there is such significant release at pH 6.5 and 5.5 that it is hard to argue that there would be a clinically significant difference between the release rate at pH 6.5 and lower pH.

Figure 4.19 shows the release data for chlorhexidine diacetate. Here, a textbook example of ideal pH controlled release is observed. The release is very rapid at pH 4.5, while at pH 6.5 there is a barely detectable release of drug. At pH 5.5, the release appears to be linear with time.

Figure 4.20 shows the absolute cumulative release of tetracycline HCl. As can be seen from this plot, the absolute release is very slight for microspheres that probably contain more than 4% drug. There appears to be little statistically significant difference in release rates as a function of pH. The release rate at pH 4.5 is arguably higher, but not enough to have a practical effect.

Linear regression analysis against $t^{1/2}$. As described previously, the data, with the 6 hour time point subtracted from the cumulative release, plotted against $t^{1/2}$. By analyzing the regression data, a determination could be made as to whether or not the diffusion is Fickian.

Figures 4.21 through 4.24 show the linear regression curves for the three chlorhexidine derivatives and tetracycline HCl. Most of the curves fit, or nearly fit the linear model with a few notable exceptions. Chlorhexidine dihydrochloride at pH 4.5 had an r^2 value of 0.8958. The chlorhexidine diacetate curves at pH 4.5 and 5.5 did not fit the Fickian model. While the r^2 value for the curve for chlorhexidine diacetate at pH 5.5 was

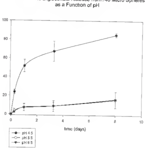


Figure 4.17: Release of chlorhexidine digluconate from T40 microspheres plotted against linear time

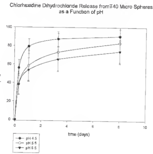


Figure 4.18: Release of chlorhexidine dihydrochloride from T40 microspheres plotted against linear time

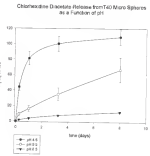


Figure 4 19 Release of chlorhexidine diacetate from T40 microspheres plotted against linear time

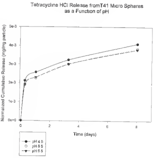


Figure 4.28. Absolute release of tetracycline hydrochloride T41 microspheres plotted against linear time

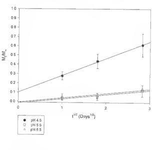


Figure 4.21 Linear regression of modified chlorhexidine digluconate release data for T40 microspheres versus $t^{1/2}$

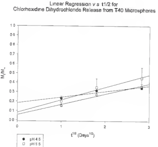


Figure 4.22: Linear regression of modified chlorhexidine dihydrochloride release data for T40 microspheres versus $t^{1/2}$

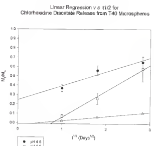


Figure 4.23 Linear regression of modified chlorhexidine diacetate release data for T40 microspheres versus $t^{1/2}$

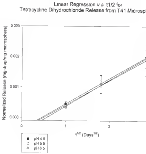


Figure 4 24 Linear regression of modified tetracycline release data for T41 microspheres versus $t^{1/2}$

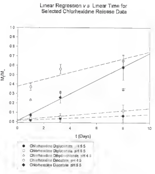


Figure 4.25. Linear regression of modified release data against linear time for chlorhexidine diacetate at pH 4.5 and 5.5, chlorhexidine digluconate at pH 5.5 and 6.5, and chlorhexidine dihydrochloride at pH 4.5

good, the intercept was strongly negative indicating a positive change in the slope ($d^2M_t/d^2t>0$).

Figure 4.25 shows a linear regression of chlorhexidine diacetate at pH 4.5 and 5.5, chlorhexidine digluconate at pH 5.5 and 8.5, and chlorhexidine dihydrochloride at pH 4.5. This graph shows that the release of chlorhexidine diacetate at pH 5.5 is "zero" order, or linear with time (r^2=0.9969). Similar, but less conclusive evidence is displayed for chlorhexidine digluconate at pH 5.5 (r^2= 0.9952) and pH 8.5 (r^2=.9812). With more data points it may be possible to prove this latter case also. The remaining cases had very poor fits to this linear model. Refer to Table 4.7 for relevant linear regression parameters.

Table 4.7: Linear regression analysis of selected controlled release curves versus linear time.

Drug	Polymer	pH	Intercept	Slope	r^2	0-order?
KF	T40	5.5	1521	0008	.5891	N
KF	T51	4.5	3411	0005	7090	N
Chlorhexidine digluconate	T40	5.5	0123	0064	9952	Y
Chlorhexidine digluconate	T40	6.5	0191	0123	9812	Y
Chlorhexidine dihydrochloride	T40	4.5	2388	0145	8014	N
Chlorhexidine diacetate	T40	4.5	3769	0367	8398	N
Chlorhexidine diacetate	T40	5.5	0113	0715	9989	Y

Comparison of release rates by drug type. Comparison of the release curves shows that chlorhexidine dihydrochloride released the most rapidly under the more basic conditions (pH 5.5 and 6.5). Chlorhexidine diacetate released most rapidly at pH 4.5, but was best retained at pH 8.5. The release rate of chlorhexidine diacetate at pH 5.5 was linear with time, but relatively rapid.

Mechanisms of Release

Controlled-release in the polymer systems studied here can be attributed to a number of factors. These systems are complicated ones in that there are polymer/solvent interactions superimposed upon drug/polymer interactions and drug/solvent interactions. While all of these behaviors will occur in concert, one will likely occur at a more rapid rate, or result in a greater thermodynamic change than the others. This factor can be termed the "controlling mechanism", since its effects are so great, that it hides the effects of the other factors. The controlling mechanisms in this systems can be broken into the following categories:

(1) Diffusion controlled-release. This mechanism is characterized by the Fickian nature of the release. In a plot of cumulative release versus time raised to the ½ power, the curve would appear linear.

(2) Swelling controlled-release. When this mechanism is dominant the appearance of so called "anomalous" or "case-II" release is noted. Here, the cumulative release curve does not generally follow a $t^{1/2}$ form.

(3) Chemically controlled-release is a broad term that describes drug/polymer and/or drug solvent interactions. This mechanism is not easily discernable from the swelling controlled case. In this case, however, the data may oppose the otherwise expected trend. This is also indicated when the amount of drug released from the polymer at the equivalent of infinite time (M_∞) is less than the percent loading as measured by other techniques.

In order to determine the possible mechanism of release, it is first necessary to determine the kinetics of release. In the following discussion the linear regression data will be analyzed to determine if the release is Fickian or anomalous Second in cases where anomalous release is observed, linear regression against linear t will be analyzed to determine if the release falls into the special case of "zero-order" or "case-II" diffusion Finally, a discussion of the possible controlling mechanisms behind each type of release will be explored.

Linear regression data analysis. In order to determine whether release is Fickian or anomalous it is first necessary to define the limits in which the classification of Fickian release may be assigned In cases of classical Fickian release, a nearly perfect fit to $t^{1/2}$ is expected. That is to say, that any variance from $r^2=1.0$ is suspect That being said one must take into account the variability in the data being studied and assume that the standard deviation in the data may lead to some error that causes a less than perfect fit Somewhat arbitrarily we can establish the following standards:

(1) If the intercept value is significantly less than 0, then a positive change in the slope with respect to time (d^2M_t/dt^2) is evident, and Fickian behavior may be ruled out.

(2) If the r^2 value is less than 0.95, then it is difficult to argue that Fickian behavior applies, unless the standard deviation in the data is large

By using these standards we can determine what controlled-release curves fit the Fickian model The results of this analysis are depicted in Table 4.6

"0-order" release special cases. Of the 24 situations analyzed in table 4 6, 9 were considered non-Fickian In these cases, the next step was to determine if the release fit into the special case of "0-order" release To do this, each of the 9 plots was graphed against linear time Another linear regression analysis was done on those that appeared to be likely to yield a favorable r^2 value (there was no obvious curvature in the plot)

Of the 9 micro sphere/pH combinations, 7 were re-analyzed for 0-order behavior, including chlorhexidine digluconate at pH 5.5 and 6.5, (r^2= 9813 and 9753, respectively) and chlorhexidine diacetate at pH 5 5 (r^2= 9941) The remaining four combinations were found not to fit this linear-linear model (see table 4 7)

Mechanisms According to our classification systems for most cases studied we can confidently declare that the release was due to diffusion of the drug from the polymer matrix, and that the mesh size of the matrix was sufficiently large that it did not interfere significantly with the diffusion of the drug.

This can be explained simply for the case of the fluoride loaded microspheres and microparticles Simply stated the size of the pores in the polymer matrix, even when unswelled, was large enough for the very small F^- ions to escape The pH control that was observed in the cases of T40 and T51 microspheres was due to the increase in pore size as a result of swelling under decreased pH This allowed for more rapid diffusion, but did not change the controlling mechanism of release The same can be stated for many of the chlorhexidine and the tetracycline loaded microspheres, but in this case, the pore size had to be generally much larger than that necessary to allow F^- to release In many of these cases, this behavior is compounded with the chemically controlled availability of the drug for diffusion.

An example of chemically controlled diffusion compounded with Fickian diffusion is easily found with the KF loaded microspheres. In these cases, the cumulative release at time is usually significantly less than the percent loading of fluoride in the polymer. This is most likely due to the interaction of the amine groups in the polymer with the fluoride ion. Once the unbound "excess" fluoride has eluted from the system, release stops. This is also a pH dependant phenomenon. This pH dependant behavior is most notable in the release from T4 microspheres. Of the microspheres in this study, T4 had the lowest percent loading. As a result, the release behavior occurs in a manner opposite that expected for this polymer. At pH 4.5, the polymer is well below its swelling transition point, and so is a highly charged species. Under the same conditions one can assume that the KF is dissociated into potassium and fluoride ion, allowing the negatively charged fluoride to readily stick to the polymer chain. At higher pH, such as 5.5, the charge on the backbone of the polymer is not as high, and so the fluoride escapes more easily. Under the nearly un-charged conditions at pH 6.5, fluoride release is fastest, and probably limited largely by polymer porosity. With more highly loaded microspheres such as T40 and T51 this condition is not observed, and release is controlled more by the swelling of the polymer under pH change.

The release profiles of tetracycline HCl are another challenging case. The drug in these microspheres was loaded to such a high degree, that the spheres were visibly dark brown in color. Although the actual percent loading of the microspheres was not measured due to complicating factors, one can assume (from previous experience) that the loading was well in excess of 4%. Yet the microspheres released such a small amount of drug that it was difficult to detect in solution (approximately 0.5 ppm at each sampling). When one compares this behavior to the release of a very similar saturated drug like chlorhexidine the release becomes all the more puzzling. One possibility is that while the molecular weight and size of the two molecules are comparable, the tetracycline is a multi-ring structure that is stiff and so unable to alter its shape easily. Chlorhexidine, however, is two benzene rings separated by a long chain of rotatable bonds. As a result, the chlorhexidine is able to assume conformations that effectively increase its diffusion rate in tight spaces, while tetracycline cannot. (See figure 2.1 and 2.2) The possibility of a chemical interaction between the tetracycline and the polymer is also not ruled out.

A final example of chemically modified release is the 0-order release of chlorhexidine diacetate and the rapid release of chlorhexidine HCl. In both these cases, an acidic moiety has been attached to the chlorhexidine molecule. In the presence of water, partial dissociation of these acid moieties is possible due to the association of chlorhexidine molecules in solution.[72] This dissociation will effectively lower the local pH in the polymer. This effect is readily observed in chlorhexidine dihydrochloride loaded microspheres. Figure 4.5 shows the swelling of these microspheres. At pH 4.5, the swelling is very rapid. The equilibrium swelling value far exceeds the 20% observed with the chlorhexidine digluconate loaded microspheres at this pH. In the case of the HCl modified drug, the swelling increase due to the presence of HCl is so drastic that the microspheres release all of their drug very rapidly. In the case of the acetate modified drug, however, there is a synergistic effect between the acetate and the polymer due to closeness of the pK_a of acetate to the swelling transition pH of the polymer. The exchange of hydrogen ion between the polymer and the acetate molecules leads to a well controlled swelling process at pH 5.5.

The case of chlorhexidine diacetate loaded microspheres is also an excellent example of swelling controlled release. The swelling and release curves (figures 4.3 and 4.22 respectively) for this system are remarkably similar. A Fickian curve is displayed at pH 4.5, while a baseline curve appears at pH 6.5. Both curves for pH 5.5 appear to have the same linear form. Superposition of the two curves reveals that they have nearly the same slope (figure 4.26). The observed presence of a swelling front during the swelling of these polymers, combined with the above observations leads to the conclusion that the release rate in this case is directly proportional to the swelling.

Efficacy of Microspheres in Broth *S. mutans* Culture

Chlorhexidine diacetate and digluconate microspheres were studied in a realistic *S. mutans* broth culture model. The bacterial population size was measured as a function of broth pH in a modified tryptic soy broth. The intended result of this experiment was to determine if, when the pH of the broth dropped (through the natural production of lactic acid by the bacteria) to pH 5.5 or less the bacterial population size would be decreased due to the release of chlorhexidine.

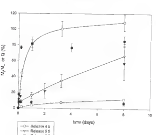


Figure 4.26: The superimposed swelling and release curves for chlorhexidine diacetate

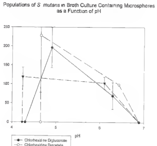


Figure 4 27: *S. mutans* populations in broth culture as a function of pH Samples including chlorhexidine diacetate and chlorhexidine digluconate microspheres are compared to microsphere free controls

Figure 4.27 shows the plot that resulted from this experiment. As can be seen, the populations of the two microsphere sample sets were indistinguishable from the controls above pH 6. At the time of the third measurement, however, the control samples had reached pH 4.26 while the microsphere samples were between pH 4.6 and 4.8. Both microsphere samples had extremely high populations (between 200 and 250 million CFU/ml) relative to the controls at this point. By the time of the fourth measurement, all populations had gone to 0. The pH of the chlorhexidine diacetate microsphere samples was stable at pH 4.6 at the time of the last measurement. The pH of the chlorhexidine digluconate samples had fallen to 4.28, indicating microbial activity between the third and the fourth measurement.

This data indicates that the chlorhexidine diacetate microspheres effectively killed the *S. mutans* population in the broth before the bacteria killed themselves by bringing the pH to an un-survivable level, as was the case with the controls. The chlorhexidine digluconate microspheres apparently did not kill the entire population within the time-frame of the experiment, as indicated by the low final pH of the broth. This does not mean that the microspheres did not release chlorhexidine, but that they did not release an effective amount of chlorhexidine rapidly enough to kill the bacteria before they were able to lower the pH to an un-survivable level. Both types of microspheres contributed to a super-infection of the broth by absorbing acid that would otherwise have killed the bacteria before they were able to reach a higher population. This probably does not constitute a drawback in clinical use, since the absorption of acid will prevent tooth decay by keeping the pH up. At the same time as the microspheres are absorbing acid, they will release chlorhexidine which in a more slowly developing population would probably reach sufficient levels to kill the microbes before they were able to reach a super-infection population level

Discussion

The focus of this work was to examine the effects of drug type on the loading release and swelling of these pH sensitive polymers, and to study the effects of these drug loaded microspheres in a relevant *in vitro model*

With fluoride microspheres, it was found that the mismatch in the hydrophobicity/philicity of the polymer drug combination can lead to lower loading. This parallels observations of other authors such as Li, *et al* who observed that the partition coefficient of drugs heavily influenced their release from polymer matrices.[4]

At low percent loading, fluoride also was found to bind to the polymer matrix and release in a manner which is opposite that expected Yan observed a phenomenon similar to this when studying sodium fluorescein in anionic hydrogels [37] Nizuka reported a similar ion-exchange phenomenon when studying the release of a cationic protein from acrylic acid hydrogels.[73] With higher percent loading, the release of fluoride followed the expected trend This was also observed by Yan in his study of the release of sodium fluorescein from anionic hydrogels [37] While fluoride release rates could be modified by the pH, and therefore by the swelling of the polymer matrix, it could not be stopped by de swelling the polymer This is to be expected since other authors have observed the controlled-release of fluoride from inorganic glasses and other polymers by a similar ion exchange mechanism [41, 48, 74, 75]

In the studies of the loading of antibiotics, it was found that the acidic moieties on chlorhexidine dihydrochloride and diacetate lead to more significant loading within the polymer matrix and the loading seemed to scale with the acidity of the ligand Both Seigel and Wei found differences in polymer swelling with pH sensitive systems when the buffering compound was changed.[33, 35] Wei found that "the swelling rate, onset swelling pH and equilibrium swelling extent were controlled by the size, lipophilicity and hydrophobicity of the acid molecules [33] In the studies of Batch and Yan, the drug and the percent drug loading was found to strongly affect both swelling and release In copolymers of styrene and diethylamino(ethyl methacrylate) (DEA) loaded with 9-amino acridine (AA) or sodium fluorescein, they found that AA loaded spheres swelled at a pH slightly above the swelling transition point of unloaded microspheres These microspheres also released AA at this pH This premature release was ascribed to AA increasing the osmotic drive for solvent to enter the microspheres Additionally the loaded microspheres were found to swell at a slower rate than unloaded ones at pH's below the swelling transition. The reduced rate of swelling was ascribed to the fact that AA is a cationic drug that would compete with the polymer for hydronium ion during swelling, thereby reducing the net concentration of hydronium available to induce swelling A decrease in swelling half-life was correlated to an increase in percent loading for these microspheres For sodium fluorescein (a highly charged dye) loaded spheres, the behavior was much more complex, and more difficult to explain At pH 7, un-swelled microspheres released fluorescein, while at pH's below the swelling transition, the microspheres did not release. While AA is a cationic drug, fluorescein is an anionic drug. The observed phenomena are likely a combination of improved solubility of the drug at pH 7 over lower pH, due to increased ionization, and binding of the anionic drug to the cationically charged polymer matrix.[37] In a similar way, the drug studied here affected the onset swelling pH, extent of swelling, and the rate of swelling in addition to modifying the release. When the acidic drug molecules were compared with relatively neutral drugs like chlorhexidine digluconate, not only was the loading lower in the latter case, but the release was generally slower with a lower onset pH. The acidic molecules were found to modify the behavior of the polymer in a controlled way which could be turned to an advantage. This is readily seen with the 0-order release kinetics of chlorhexidine diacetate at pH 5.5.

The effect of drug type was readily apparent when the rates of release of tetracycline were compared with the comparable chlorhexidine molecules. Tetracycline was found to not release to any significant extent at all. This was attributed to drug size and inflexibility limiting its ability to diffuse from the tight pore structure of the polymer matrix. Olanoff found that the diffusion coefficient of tetracycline was proportional to the percent hydration (and therefore to the mesh size) in (hydroxyethyl methacrylate)-co-(metyl methacrylate) copolymers. At a hydration of 5% the diffusion coefficient was 8 cm^2/sec x 10^{-9} versus 43 cm^2/sec x 10^{-9} for a 21% hydrated polymer. The microspheres studied here are as much as 20% water at maximum swelling.[7] This indicates either that the diffusion kinetics seen in HEMA copolymers are different than those seen in the microspheres or that there is some chemical interaction occurring within the polymer which may either prevent swelling, or bind the drug such that it does not release. The possibility that the tetracycline was degraded at the time of release remains, but is unlikely since the aqueous controls run alongside the experimental group were not degraded until near the end of the study.

The *in vitro* experiment conducted with *S. mutans* in modified tryptic soy broth showed that chlorhexidine diacetate microspheres effectively killed the bacteria at a pH below 5.5. The data regarding the efficacy of chlorhexidine digluconate loaded microspheres was inconclusive. In a similar study, Shen observed that styrene-co-diethylamino(methyl methacrylate) microspheres loaded with clotrimazole or nystatin effectively killed *C. albicans* in a plate culture. He observed that the fungi frequently grew over the nystatin loaded microspheres before a zone of inhibition formed. The similar phenomenon of a super-infection was observed in the case of *S. mutans* in broth culture.

CHAPTER 5
CONCLUSIONS

In this study the loading, swelling, release and efficacy of 5 different drugs loaded into a single polymeric system were examined. In addition the loading into and release from various copolymer systems of one model drug was studied The following conclusions can be drawn

Synthesis

(1) Synthesis of fluoride loaded micro spheres with fluoride included as part of the monomer mixture proved difficult

(2) Synthesis of micro particles with intrinsically loaded drug was simple and yielded good quality particles

Loading

(1) The percent loading of fluoride microspheres was low due to the incompatibility of the hydrophilic fluoride with the hydrophobic polymer

(2) Drugs with highly acidic ligands loaded according to the degree of acidity of the moiety, greater acidity leading to greater loading

Swelling

(1) Microspheres swelling was heavily controlled by the drug which was loaded into it.

(2) Drugs with acidic moieties increased the equilibrium swelling, increased the swelling transition pH, and increased the rate of swelling with reference to an identical, but neutral drug. The degree of change was more pronounced with acidic moieties with lower acid constants.

Release

(1) With few exceptions, fluoride released in a Fickian manner regardless of the pH. This is due to the small size of the fluoride ion, allowing it to diffuse in and out of the polymer nearly at will.

(2) Chlorhexidine hydrochloride released very rapidly, regardless of pH. This is probably due to excessive swelling induced by the presence of the hydrochloride group in the drug.

(3) Chlorhexidine digluconate was well retained at pH 5.5 and 6.5, but release at pH 4.5 was Fickian. At a pH between 4.5 and 5.5, 0-order release might be expected.

(4) Chlorhexidine diacetate was well retained at pH 6.5, released rapidly at pH 4.5, and released at a rate linear with time at pH 5.5. This 0-order release

rate was probably swelling controlled. The acetate group on the drug contributes to the swelling behavior of this drug/polymer combination.

(5) Tetracycline released very little, and was not pH controlled to a significant degree. This is probably due to chemical interactions with the polymer which either prevent swelling, or lead to the binding of the drug to the polymer matrix.

(6) pH controlled release was heavily influenced by the ligands attached to the drug molecules, leading to drastically different behaviors even when the root molecule remained constant.

(7) The drug release rate correlated with the swelling observed in drug loaded microspheres.

Efficacy in Broth *S. mutans* Culture

(1) Chlorhexidine diacetate microspheres effectively killed *S. mutans* on a pH controlled basis. The results for chlorhexidine digluconate loaded microspheres are inconclusive.

(2) Microspheres promoted a super-infection in the broth culture before killing the population, when compared with the no-microsphere controls.

CHAPTER 6
FUTURE WORK

The results of these studies have pointed toward a number of experiments which would further advance both the development of this specific technology, as well as the field as a whole.

Synthesis and Loading

(1) Micro particles proved to be very simple to make, and the size of the particles was relatively easy to control. While the studies here did not show any significant results with regard to controlled release, with a different drug loaded into the particles, excellent behavior may be observed. Micro particles should be made, and then post-loaded with drug by methods similar to those used for micro spheres. High temperature initiators, or low temperature/long polymerization conditions should be used for a more controllable polymerization.

(2) Fluoride proved to be too small, and the wrong ionic charge to allow for controlled release from these polymer systems. It is unlikely that any system will ever provide on-demand release of fluoride unless the ions are bound to a high molecular weight matrix. Work with this ion should be

discontinued, unless a continuous slow release regardless of pH, is what is desired

Release

(1) The release of chlorhexidine diacetate in citrate buffers was a perfect 0 order case. This system should be tried in different buffer systems to see if it is exclusively a pH controlled phenomenon.

(2) The acetate molecule seemed to moderate the swelling behavior of the polymers studied It would be advantageous to test the swelling of other polymer systems with acetate loaded into the matrix To study the release of other drugs from the polymer studied here, with acetate loaded into the matrix as a co-drug would also be a productive avenue

Bacteriological Evaluation

(1) This model was successful, but the number and variety of samples was small This protocol should be repeated with a larger sample size more frequent sampling, and with more test samples

(2) The model presented here is an excellent first screening test, but is not clinically relevant to any significant extent. A model similar to Dr Shen's micro leakage model[2] employing the use of these micro spheres should be attempted The micro spheres could be placed below dental restorations

in extracted teeth which have been inoculated with a cocktail of cariogenic bacteria. After a defined time in a nutrient broth, the restorations could be extracted and bacterial viability assessed

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BIOGRAPHICAL SKETCH

John Wironen grew up in New England as the youngest member in a family of nine. He attended the University of Massachusetts at Amherst where he attained a degree in physics while also studying pre-medicine. After college, John spent a year working as a research technician in the Department of Nuclear Cardiology at the University of Massachusetts Medical Center, Worcester, MA.

In Gainesville, he has conducted research in orthopedic biomaterials, bacterial adhesion to surfaces, and in controlled release technology.

When not working, John has spent many rewarding hours during the last four years, spending time with his "little brother" through Big Brothers and Big Sisters of Gainesville. He enjoys spending time with his family in New England, and remains an avid fisherman.

John hopes to continue his research into biomedical materials as the Senior Scientist at the University of Florida Tissue Bank, Inc., in Alachua.

APPENDIX PUBLICATIONS

1 Wironen JF, Kao PE Dohse S, Strates BS The Effect of Bone Protein and Carrier Matrices on BMP-Stimulated Osteogenesis *44th Annual Meeting Orthopoedic Research Society* (New Orleans, LA, March 16-19, 1998) (Pending Abstract)

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy

Chris Batich

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy

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Associate Professor of Materials Science
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy

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